BIOPHYSICAL CHARACTERIZATION AND BIOPROCESSING OF PROTEINS

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the Requirements for the Degree Doctor of Philosophy

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TITLE: Biophysical characterization and bioprocessing of proteins

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DOCTORAL DISSERTATION

NUMBER OF PAGES: xix, 132

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ABSTRACT

Advances in biotechnology have opened up numerous new avenues for researchers in their study of proteins. These new technologies have not only resulted in a more robust understanding of protein characteristics which had hitherto only been achievable through the use of a handful of techniques - but they have also made this knowledge accessible to a wider audience. Although traditional approaches to studying proteins have performance limitations, they have remained the preferred options due to the absence of better alternatives that can accommodate similar scales of operation. Ideally such scenarios are commonly encountered during bioprocessing, which involves the extraction and purification of a pure protein from a host cell organism via the separation and removal of impurities. Here, the protein of interest undergoes thorough treatment to remove unwanted impure substances while at the same time preserving the targeted qualities.

This thesis introduces approaches to protein characterization and bioprocessing and discusses their limitations. In addition, newer alternatives are also introduced, particularly those that provide improved efficiency and quality of information compared to the traditional approaches. Specifically, this thesis highlights various aspects of bioprocessing and the biophysical characterization of proteins, with a focus on areas such as the processing of aggregates, impurity detection in protein samples, and the structural stability of proteins.

Overall, the results presented in this thesis were obtained with respect to the specific protein(s) under consideration. However, the nature of the presented processing techniques enables their use in the study of other similar proteins. As such, the results presented herein are not necessarily limited to a specific protein type rather, they can be generalized for application to a wide range of proteins, thus highlighting the true potential of these techniques. Finally, recommendations are made with regards to further exploring the wide scale application of these new approaches. In general, the new approaches presented in this thesis do not involve complex mechanisms; on the contrary, they are relatively easy to understand and perform.

This thesis covers the following points and presents preliminary data supporting the hypotheses:

(i) The problem of protein aggregation is very common in the bioprocessing industry and solution(s) to remedy this problem typically involves tedious and energy intensive approaches. In this thesis, a simple and easier to perform thermal-cycling method capable of disaggregating protein clusters and refolding the resultant isolated protein chains into functional protein molecules is presented and explained.

(ii) Another aspect of bioprocessing examined in this thesis is the impact of prolonged interaction time between a protein and substrate post adsorption. During the study it was observed that prolonged contact between a protein and adsorptive surface causes structural changes in proteins. This change was identified based on chromatographic peak broadening within an interval of 10-15 minutes.

(iii) Heterogeneity in proteins is introduced during processing and storage, and this can adversely impact the quality of the target product. Using a rapid analytical membrane chromatographic technique, it was possible to identify monoclonal antibody charge variants. While the obtained resolution was comparable to that of column chromatography, membrane chromatography provided faster processing overall.

The work documented in this thesis provides a "proof-of-concept" methodology as an alternative to currently used protein analysis techniques. The new methods presented in this thesis lay out a platform for the further investigation of these ideas. There is immense scope for thorough and detailed studies to be performed to further bolster the principles discussed herein. Since the presented data is only a preliminary finding with a single test protein, more elaborate testing is required to validate the proposed hypotheses of this work.

ACKNOWLEDGEMENTS

My journey until completion of this thesis has been fruitful, filled with challenges and intellectually satisfying. This would not be possible without the guidance and support of people involved, directly or indirectly. First and foremost, my sincere gratitude and thanks to my supervisors Dr. Raja Ghosh and Dr. Carlos Filipe for their constant encouragement - who believed in me and allowed me to pursue my research interests. I am especially thankful to Dr. Raja Ghosh and Dr. Christopher Swartz (Dean of Graduate Studies - 2013) for giving me the opportunity to pursue doctoral research after completing my Masters' degree.

To my supervisory committee members, Drs. Richard Epand and Prashant Mhaskar, thank you for the valuable inputs and guidance. Your comments and criticisms during the meetings have further improved the quality of this thesis. Thanks to the departmental staff over the years including Kathy Goodram, Lynn Falkiner, Cathie Roberts, Melissa Vasil, Michelle Whalen, Kristina Trollip, Paul Gatt, Justyna Derkach, Doug Keller, Tim Stephens, (Late) Dan Wright and Mike Clarke for helping with administrative and technical difficulties.

I would also like to acknowledge the support and friendship from members of the McMaster Bioseparations Engineering Research Group (MBERG): Drs. Seungmi, Xiaojiao (Annabel), Pedram, Pan Si and Guoqiang Chen. Special thanks to MBERG alumni Drs. Dharmesh and Mukesh for their invaluable words of wisdom throughout my

research. I would also like to appreciate support and friendship from visiting scholar Qijiayu Wu. At McMaster my friends Avijit, Ish, Abhinav, Smriti, Ameya and Pulkit have provided immense support and great company.

During my graduate research, I also got the opportunity to interact with my friends - Abhiram and Krunal and Purnendu with whom I had interesting discussions on therapeutic protein development and topics related to bioprocessing.

I want to specially thank my family and in-laws who have stood behind me providing the necessary support and who also had to deal with my intense arguments. Lastly, I would also want to thank my wife Mitushi Sadavarte who believed in and helped me sail through the tough times during the final stages of writing this thesis.

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LIST OF ABBREVIATIONS

ANT	Aminoglycoside Nucleotidyl Transferase
BL21(DE3)	Competent Cells Suitable for Expression of Heterologous Genes
CD	Circular Dichroism Spectroscopy
CD4	Cluster of Differentiation 4
CE	Capillary Electrophoresis
CEX	Cation Exchange Chromatography
DLS	Dynamic Light Scattering
DTT	Dithiothreitol
E. coli	Escherichia coli
EDTA	Ethylene Diamine Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
Fab	Antigen Binding Fragment of Antibody
Fc	Crystallizable Fragment of Antibody
FcRn	Neonatal Fc receptor
FF	Fast Flow
HIC	Hydrophobic Interaction Chromatography
hIgG	Human Immunoglobulin G
HP	High Performance
HPIEC	High Performance Ion Exchange Chromatography
IB	Inclusion Body
ICH	International Conference on Harmonization
IEF	Isoelectric Focusing
IEX	Ion Exchange Chromatography

IPTG	Isopropyl-β-D-ThioGalactopyranoside
kDa	Kilo Dalton
LB	Lysogeny Broth
LFMC	Laterally-Fed Membrane Chromatography
mAb	Monoclonal Antibody
NIBS	Non-Invasive Back Scattering
PDI	Polydispersity Index
PEG	Polyethylene Glycol
pH	Concentration of hydrogen ions
pI	Isoelectric Point
RP-HPLC	Reverse Phase-High Performance Liquid Chromatography
S	Sulfonated
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SP	SulfoPropyl
V	Volts

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Currently, the spread of life-threatening infectious diseases (e.g., COVID-19) and cancers are a significant problem globally resulting in the deaths of millions of people. At this critical juncture, the production of therapeutic proteins for the treatment of such diseases has become a priority. Thanks to advancements in recombinant DNA technology, the mass production of proteins such as vaccines, diagnostic markers, therapeutics and small chain peptides, is now achievable in short periods of time.[1,2] These recombinant proteins are mainly produced using either prokaryotic or eukaryotic hosts under controlled conditions in a bioreactor.

Various expression systems are available for recombinant protein production, such as mammalian cells, bacteria, yeast, insect cells, as well as transgenic plants and animals. Depending on the application requirements, recombinant proteins can either be obtained at laboratory- or production-scale quantities. An efficient bioprocessing strategy is critical to achieving the end goal of producing a specific protein as downstream bioprocessing often presents a bottleneck in the handling of large protein feed streams and requires the custom tailoring of the purification and processing steps to achieve the desired product quality. This requirement has motivated intense research aimed at understanding how the various purification and processing steps impact protein production and quality attributes, and, accordingly, which strategies can enable the smooth transition and handling of feed streams.[1-3]

At present, researchers are exploring the use of high-throughput and continuous processing, along with single-use disposable systems, to achieve efficient and faster bioprocessing. While the processing stages take care of the workflow and transition of protein feeds between different steps, it is equally important to analyze and understand the product quality obtained at the end of each processing stage. This is because proteins are marginally stable amphiphilic molecules with large peptide chains that are affected by changes in the properties of the solution.[4,5]

During processing, the equilibrium state of the proteins is altered by changes in the solution conditions and interaction with the purification medium, which destabilizes the peptide chains and causes them to stick to each other, forming a cluster of molecules. This process is known as "aggregation."[4,6,7] Since aggregation is encountered during every step of protein processing, much work has focused on developing strategies to thwart the formation of such aggregates.[8-10] In certain special cases, the intentional formation of protein aggregates is desirable, as it can increase protein yield and recovery.[11-13] The resultant aggregated masses are then further treated to obtain functional protein molecules. At the same time, the goal of any protein production process is to obtain a pool of homogenous protein molecules – however this is challenging because the protein surface may be altered during both the expression and purification stages.[14,15] This introduction chapter highlights recent developments in the bioprocessing of recombinant proteins, including the use of various expression systems and, protein processing strategies, as well as approaches to characterizing the protein product.

1.2. Bioprocessing and Recombinant Technology

Therapeutic proteins and peptides are the main drugs developed in the biopharmaceutical sector.[1-3] The demand for protein-based pharmaceutical products experienced a massive increase after the successful introduction of recombinant insulin (Humulin®) in 1982,[16,17] with numerous recombinant proteins ranging from human growth hormone (rhGH) to vaccines having since been approved for human use by regulatory agencies across the world.[18,19] Indeed, at present, 100 protein-based and 100 peptide therapeutics have either received FDA approval or are undergoing clinical trials, with an additional 500 currently in the preclinical development phase. Overall, the market value for biologics is estimated to be greater than $250 billion,[20] consisting of more than two hundred therapeutics currently available for commercial use.

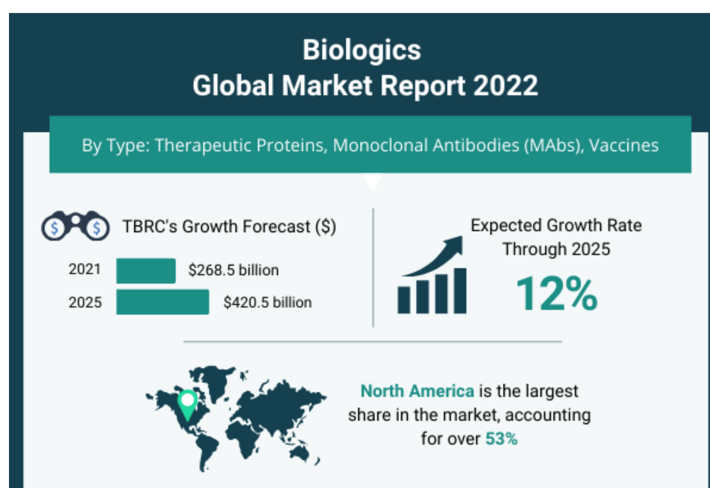

Figure 1.1: Biologics Global Market Report of 2022.[8]

1.3. Evolution of Recombinant Technology

Since its invention in the early 1970s,[18,20] recombinant DNA technology has attracted the attention of researchers and large corporations, mainly for its potential to generate large profits. The unique ability to alter the genetic material in a host cell, thereby converting it into a small individual factory able to continuously churn out a target protein product seemed very attractive.[2,3,18,19] The ability to manipulate genes in this manner made it possible to obtain chemicals of significance to areas such as science, medicine and agriculture. In the beginning, recombinant DNA technology appeared risky due to the lack of evidence supporting the feasibility of this radical approach and the ability to produce a market-ready product in sufficiently large quantities. Despite these concerns, this new technology piqued the interest of many investors.

1.4. Protein Expression Using Host Organisms

During the early stages of the development of recombinant technology, the production of large quantities of target protein incurred huge capital costs, an outcome which was not wholly unexpected. However, with the gradual advancement of this technology, it became possible to obtain higher protein yields at significantly lower operational costs, thus enabling easier access to therapeutics for the treatment of life-threatening diseases and disorders. To meet the demand for high-value and high-potency therapeutics, it is necessary to thoroughly screen various expression hosts and technologies for protein extraction and purification, such as plant, animal, mammal, bacteria, yeast and insect cells. Among the

various available options, the most commonly used host organism is the bacterium *E. coli*.[18,19,21] Yeast cells (*Saccharomyces cerevisiae* and *Pichia pastoris*) are another common expression system used for the manufacturing of proteins, as they can produce a target protein with the desired post-translational modifications (PTMs).[21] Similarly, mammalian cell lines are an attractive choice for achieving large-scale industrial protein production[21,22] due to their ability to allow robust growth in well-defined cell culture media. As a result, mammalian cells are widely used to manufacture the majority of commercially approved recombinant therapeutics, including Trastuzumab (HERCEPTIN®), Bevacizumab (AVASTIN®) and Rituximab (RITUXAN®) among others.

When produced as soluble entities, recombinant proteins retain their therapeutic properties and functionality, although this outcome is not always achievable.[18,23,24] Specifically, when proteins are recombinantly produced for human application, they must contain the required pharmacokinetic and physicochemical properties.[24] Therefore, the goal in choosing a particular expression technique is to achieve high titers of the soluble protein.[18,21,23,24] However, increasing the yield poses a challenge since many of these biochemically-active proteins belong to the kinase, phosphatase, membrane-associated protein or enzyme families making them difficult to produce as soluble molecules.[21,23,25,26] In such cases, recombinantly expressed proteins are extracted as insoluble aggregates and later processed to obtain soluble, functional, and biochemically active molecules.

1.5. Bioreactor Operation and Protein Production

The first step in protein expression is the selection of a cell line or host system that has been transfected and transformed with appropriate plasmid DNA fragments to produce the desired protein. Next, small colonies of the chosen cells are cultured under controlled conditions using microtiter plates, test tubes, tissue culture flasks, and shaker flasks for the initial and rapid screening of recombinant protein expression. At this stage of operation, various parameters and their effect on protein expression levels are screened, including the type of growth medium and its composition, agitation speed, aeration levels, the pH and temperature of the medium, cell density, concentration of inducer, induction time and feeding strategies, and the volume of the growth medium.[18,19,21,23,27] The complexity arising from these operational parameters makes it extremely important to thoroughly study their effect on recombinant protein expression, as this can be a critical step in helping to develop an effective bioprocessing system.

For thorough optimization and screening of the growth conditions, high-throughput process development (HTPD) techniques are implemented[28] to improve process efficiency and reduce production costs. In addition, researchers have also examined operations such as single-use upstream and downstream processing for their ability to reduce the costs and process time associated with recombinant protein production.[29] Once the parameters for cell growth are finalized, large-scale protein production using bioreactors is then applied to meet the production targets.

Bioreactors are hollow vessels in which the growth of a microorganism, also known as the fermentation process, can be carried out in a controlled environment. **Figure 1.2** shows a typical bioreactor, along with the various components that are critical to its functioning. A comprehensive review of the various types of bioreactors along with their advantages and limitations is presented elsewhere.[30] In selecting a bioreactor, it is important to understand the key features affecting it performance. The various key features essential for an ideal bioreactor system have been detailed in a previous reference article.[31]

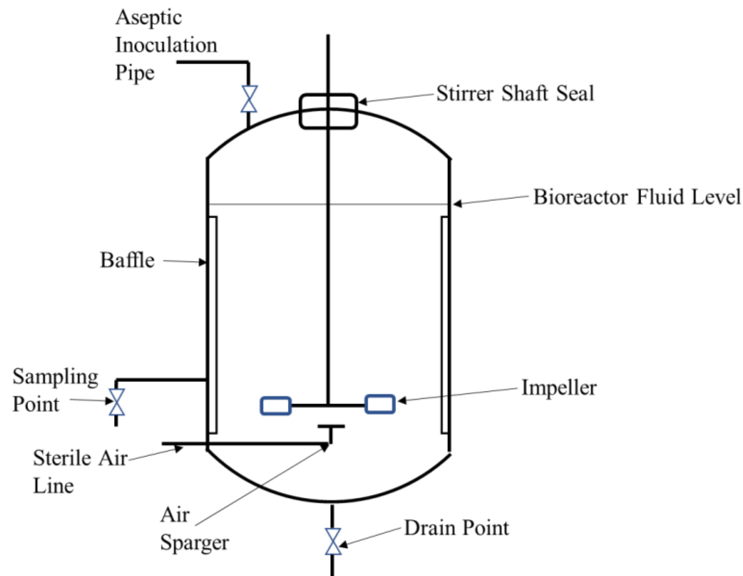

Figure 1.2: Image showing key components of a bioreactor.

Depending on the nature of the system, bioreactors can be operated in a number of different modes (i.e., batch, fed batch and continuous culture) for the bulk production of recombinant proteins. Among these modes, the batch and fed-batch processes are very similar, with the only difference being that the fed-batch process requires the periodic addition of fresh

media and the removal of the bioreactor's contents at the end of a fermentation cycle, followed by transportation for downstream processing. Given their simplicity, these modes are the preferred options in the bioprocessing industry. The continuous mode is an alternative technique for cell culturing that eliminates the need to shut down the fermentation process. In this mode, fresh growth media is continuously added to the reactor and bioreactor fluid is withdrawn at the same time. The simultaneous addition and withdrawal of media from the bioreactor helps the cells receive fresh growth medium and prevents the buildup of toxic cell waste.[20] Continuous bioprocessing is also advantageous because it requires smaller facilities, exerts a lower equipment footprint, and facilitates rapid process development and process scale up.[33,34] The introduction of this newer technology has improved cost effectiveness in manufacturing and production, enhanced flexibility in manufacturing processes, and enabled stricter quality checks.

1.6. Selecting the Right Host for Protein Expression

Selecting the right expression host is extremely crucial to obtaining the desired protein yields and quality targets.[21,26] The selection of a poor host can result in misfolded protein, reduced expression levels, and incomplete post-translational modifications, which can ultimately lead to the production of defective and non-functional proteins. Additionally, it is also important to consider the size of the protein when selecting the expression host. Other important factors to consider include the number of disulfide bonds, the extent of post-translational modifications required on the protein surface, and the destination of the

expressed protein. As mentioned earlier, there are a variety of available expression hosts, with many having been successfully used to produce recombinant proteins. While some of these host systems are still evolving and being studied, others, such as transgenic animals, are too complicated, expensive, and challenging to handle. Given the continuous increase in demand for proteins, it is vital to choose a simpler expression system.

1.7. Escherichia coli (*E. coli*) and Inclusion Bodies (IBs)

Bacterial cells, especially *E. coli*, are fast-growing and robust organisms with the unique ability to quickly adapt to their growth environment. In addition, bacterial cells offer the advantages of fast growth kinetics and biomass accumulation, low maintenance, high cell densities, relatively inexpensive media components, ease of cell transformation, low operational costs, well-known biochemistry and genetics, and good productivity.[18,19,21,23]

The use of bacterial cells enables recombinant protein expression either in the cytoplasm, the periplasm, or the secretion of proteins outside the cell. However, a major drawback of using *E. coli* cells for protein expression is the frequent lack of proper post-translational modifications (PTMs) due to the bacterial cells' lack of the necessary mechanism to chemically alter the protein surface. This limitation restricts the widespread use of bacterial cells for protein expression. For example, proper glycosylation is required for the correct functioning of therapeutic proteins used in human applications. Glycosylation is an important characteristic in deciding the efficacy, serum half-life, and antigenicity of a recombinant biopharmaceutical.[35,36] Therefore, in such cases, alternate expression host

systems, such as mammalian, yeast, and insect cells, are preferred, as they are genetically engineered to produce a human-like glycan pattern in recombinant proteins.[21,23] Two major disadvantages of using *E. coli* cells in protein expression are inclusion body (IB) formation and endotoxin issues.[18,37]

Inclusion Bodies are formed when recombinant proteins expressed in bacteria aggregate to form insoluble protein clusters. Inclusion Bodies which were originally considered a waste product generated by the bacterial cells in response to a stress reaction, are essentially biologically inactive and precipitated protein masses. The quest to achieve high yields of recombinantly expressed heterologous proteins using bacterial systems has led the researchers to explore new and innovative strategies which are categorized into different groups as detailed elsewhere.[38,39] The strategies presented share a common goal: the challenge of improving protein solubility and purifying the expressed protein.

Findings have revealed that 80% of recombinant proteins, when overexpressed in *E. coli* cells, still form Inclusion bodies.[40] While this high rate of failure to obtain pure protein would traditionally disqualify the use of a method, over 30% of biopharmaceuticals currently being manufactured and distributed still use *E. coli* expression systems.[41] The continued use of *E. coli* expression systems is due to the attractive features of Inclusion bodies, including ease of isolation due to their density and size differences compared with the host cell proteins, their resistance to degradation by host cell proteases, their mechanical stability, their native-like secondary protein structure, and their residual biological activity.[42,43] The attractive features of bacterially generated Inclusion bodies have led researchers to explore methods of improving protein solubility and stability, including the

use of molecular tags and additives, with many of these studies yielding successful results.[44-47] In addition, soluble proteins have also been successfully obtained using methods in which protein expression is induced at lower temperatures.[48-52] This approach not only enhances protein solubility, but it also allows the proper folding of the protein molecules.

Despite the disadvantages resulting from the formation of Inclusion bodies, there are some advantages, which have attracted the attention of many researchers. In particular, the protection offered against the host cell's proteases is beneficial for the individual protein chains if they are part of the Inclusion body mass. Since the benefits associated with Inclusion body formation outweigh the drawbacks, most research still centers around Inclusion body formation and further treating these aggregates to obtain functional protein molecules.

1.8. Separation and Processing of Inclusion Bodies

Recombinant proteins expressed in *E. coli* cells need to be purified in order to obtain a final product. As in every other protein production process, recombinant proteins must also undergo elaborate downstream processing. The goal of a downstream process is to obtain a high purity protein with minimal processing time and cost. The first step after the fermentation reaction is to separate the biomass containing the expressed protein from the fermentation broth, which is usually achieved via centrifugation or filtration techniques. Once the biomass has been collected, it is subjected to powerful cell-disrupting forces.

Depending on the scale of the operation, bacterial cell disruption can be carried out using high-pressure homogenization, bead milling, ultrasonication, osmotic shock, freeze-thaw cycling, and enzymatic and chemical lysis.[53,54] Mechanical disruption techniques such as high-pressure homogenization and milling are preferred for large-scale processes, while ultrasonication is the preferred method for laboratory-scale processes.[53]

After cell-membrane disruption, the high yield of protein can be solubilized, as the majority of these protein chains are either misfolded or unfolded when present as Inclusion bodies; at this point, the challenge becomes the inducement of biological activity in these proteins after refolding.[38,39,54,55] The refolding of proteins has always been a challenging task, as every protein has a distinct set of amino acids that responds uniquely under given physiological conditions.

In the case of recombinant proteins expressed as Inclusion bodies, the solubilization of the aggregated protein chains is performed with high concentrations of a denaturant (6-8 M) such as urea and guanidinium hydrochloride (GdnHCl), and detergents like sodium dodecyl sulfate (SDS), in the presence of reducing agents like dithiothreitol (DTT), β-mercaptoethanol, or cysteine. The refolding of the solubilized protein chains requires controlled removal of the denaturant and additives. Currently, protein refolding is conducted using numerous strategies involving techniques such as dilution, diafiltration using an ultrafiltration membrane, dialysis, on-column chromatographic refolding, and high hydrostatic pressure.[56,57]

Chapter Two of this thesis provides a detailed discussion of the formation of Inclusion bodies, the experimental procedures used to extract, purify, and process them, and the novel thermal-cycling procedure that is employed for further processing to obtain a soluble functional protein.

1.9. Background on Proteins

Three types of macromolecules namely – proteins, nucleic acids, and carbohydrates are responsible for many aspects of life processes. Nucleic acids, including RNA and DNA, encode and express genetic information; carbohydrates are energy molecules that are stored in living organisms in various forms; and proteins, the most abundant macromolecules in any living organism, are responsible for carrying out most of the cellular functions. Proteins are made of linear chains of amino acids linked together by peptide bonds.

In nature, there are 20 different amino acids, each containing a unique set of physicochemical properties, such as shape and electric charge, that produce attractive and repulsive forces. Historically, proteins evolved in aqueous environments inside the cells of living organisms. As such, proteins exist as water soluble molecules, which help them diffuse freely without participating in non-specific protein interactions. When a long strand of amino acids is formed, the attractive and repulsive forces drive the protein chain to collapse into a three-dimensional structure.[58-62] The presence of charges on the surface helps the protein chain to fold correctly, with polar residues located on the protein surface and non-polar residues largely buried in the core. Depending on the strength of the

interaction forces, some protein molecules remain stable while others are more flexible and rearrange themselves into different shapes.[63,64] Proteins play a role in various functions, including energy transfer, catalysis, respiration, cellular communication, photosynthesis, molecular transport, and cellular boundary, to name a few.

1.10. Protein Instability and Aggregation

The advent of recombinant technology has made it possible to manufacture commercial proteins at larger scales. However, the degradation of proteins remains a common challenge associated with this process. Of the various degradation pathways, aggregation is the leading cause of protein loss during bioprocessing.[65] Aggregation takes place throughout the lifecycle of a protein and is of great concern in the biotechnology community.[66] The problem of protein aggregation arises during expression, the purification and polishing stages, as well as during storage and handling.[66] Although several strategies have been devised to mitigate this problem, the issue continues to persist.

In common terminology, aggregation refers to the "formation of a cluster" from individual entities. Generally, researchers utilize working definitions of "aggregates" due to the lack of a standardized description of these structures. For instance, some researchers define aggregates as "particles of a specific size which cannot be removed by a certain filter size," while others define them as "protein species of higher molecular weight such as oligomers or multimers." Irrespective of the selected definition, it is generally agreed that aggregates are phenomena that arise due to instability, encompass several types of interactions, such

as physical and chemical instabilities; and are one of the major barriers to large-scale protein manufacturing.[67,68]

Aggregates can be classified as either soluble-insoluble, reversible-irreversible, physical-chemical, and native-denatured. In addition, aggregates can be categorized as either intrinsic (primary, secondary, tertiary, or quaternary structures) or extrinsic (solution environment, processing conditions, etc.). Usually aggregates of any kind are troublesome, and their presence in a protein solution is considered unacceptable.[65,66] In the case of protein therapeutics, the administration of doses containing aggregates is known to induce immunogenic reactions in patients, but it can cause adverse reactions as well.[69-71] Diseases such as Alzheimer's, Parkinson's, Amyotrophic Lateral Sclerosis (ALS), Dementia, Huntington's, Prion, Sickle Cell Anemia, Down's Syndrome, and Cataract are all the result of protein aggregation.[72-74]

Inside the host cell of an organism, protein aggregates form insoluble deposits, which results in the loss of cellular function,[75,77] the deterioration of protein quality,[76,77] and proves toxic[76,77] for the cell's survival. On the other hand, *in vitro* protein aggregates produce changes in the solution's properties and results in the loss of valuable resources. Protein aggregates can be categorized as the product of either physical or chemical aggregation, as these are the two types of aggregation which are most observed in solution.

Protein aggregates can form through different mechanisms and pathways. The major pathways include: (i) aggregation through unfolding,[78,79] and, (ii) aggregation through protein-protein self-interaction.[6,7,78] Proteins are marginally stable molecules under

physiological conditions and exist in equilibrium with partially unfolded and completely unfolded or denatured molecules.[4] When the unfolded protein chains become overpopulated, protein-protein interactions increase and unfolded aggregates are produced.[4,6,78] This phenomenon is observed most often in the bioprocessing industry during each step of protein production, as hydrophobic fractions of the unfolded chains are exposed to the aqueous environment, which further leads to higher instability.[4,6,7,78] On the other hand, the formation of dimers, trimers, tetramers, and high order aggregates occurs as the strength of protein-protein aggregates increases.[4,6,7,78] Typically, protein aggregates begin with the formation of soluble clusters and eventually precipitate out of the solution. Protein stability is impacted by several factors including additives,[80] pH,[81,8] ionic strength,[82,9] and temperature.[78,85]

The use of co-solutes has been shown to provide considerable success in countering the problems of protein instability and unfolding.[10,84,88] However, despite these results, unfolding remains prevalent. One area that needs more attention is the chromatographic interaction between the protein and separation media, as chromatography is a widely used technique in the bioprocessing industry. In chromatography, the protein of interest selectively interacts with the media components and is processed and purified from impurities present in the feed solution. Several types of interactions can be utilized to achieve protein capture, such as ion-exchange, protein-A affinity, hydrophobic interaction, and size exclusion.

Chapter Three of this thesis provides insights into the importance of the interaction time between the protein and chromatographic substrate and highlights the impact of such interaction on the resultant protein's structural stability.

1.11. Protein Charge Heterogeneity

The use of recombinant technology has enabled the custom tailoring of genes in microorganisms to obtain proteins of interest in large quantities. With the availability of such a sophisticated technique, one can expect a homogenous pool of proteins with uniform properties. While this assumption seems viable theoretically, it is far from being true in practice. In fact, in most cases, the final proteins obtained via recombinant expression display a variety of posttranslational modifications. These variations result due to changes in amino acid residues in the protein chain, as well as incorrect posttranslational modifications.[86-88] Various posttranslational modifications also occur during protein processing and long-term storage.

Most observed protein modifications include the deamidation of asparagine, the isomerization of aspartic acid, the carboxylation of glutamic acid, and the oxidation of methionine. Such modifications are known to alter the expected biological activity of the final protein product, induce illicit immunogenic responses, create a heterogeneous pool of protein molecules, and generally pose challenges during protein purification. These changes can occur during cell culture processes, downstream protein processing, and during long-term protein storage due to enzymatic and-/or chemical activity.[89,90]

Additionally, in case of monoclonal antibodies, it has been observed that the presence of charge variants can alter the structure of the native protein, which eventually compromises the efficacy and safety of the final drug product.[91] Although efforts to minimize variations in charge heterogeneity have been marginally successful with upstream modifications,[92,93] additional treatment during downstream processing is often employed to separate out protein charge variants produced during purification and storage.

On a macroscopic scale, protein variants can be defined as molecules with different surface charges under physiological conditions. This charge heterogeneity in protein species is very common among monoclonal antibodies, and the different charged species can be categorized into acidic and basic variants along with the main variants. Since the main characteristic of differentiation is the presence of differing charges, several charge-based techniques are used to identify and separate these protein variants. The most used separation techniques are ion-exchange chromatography, charge-based ultrafiltration, isoelectric focusing, and electrophoretic methods.[94]

Chapter Four of this thesis presents a new chromatographic approach for identifying and fractionating monoclonal antibody charge variants and discusses its use for the analytical testing of proteins.

This thesis is written and presented in a sandwich style based on the manuscript in progress and prior published work as described below:

Chapter One provides an overview of the significance of recombinant technology in protein manufacturing and discusses various aspects of protein bioprocessing, including bioreactor operation and protein purification. The advantages and disadvantages of properly folded protein are also briefly addressed, and the need to generate specific protein aggregates, also known as Inclusion bodies (IBs) is highlighted. Furthermore, other important aspects in biotechnology (i.e., protein processing and characterization) are also discussed in this chapter.

Chapter Two details the effects of the thermal-cycling technique on protein aggregates and how it can be applied to refold proteins.

- "R. Sadavarte, C.D.M. Filipe, R. Ghosh. Recovery of Functionally-Active Protein from Inclusion Bodies Using a Thermal-Cycling Method. Biotechnol. Prog. 33 (1) 133-139."

Chapter Three illustrates how the interaction time between proteins and chromatographic media affects the structural stability of proteins.

- "R. Sadavarte, C.D.M. Filipe, R. Ghosh. Effect of On-Column Contact Time on Protein Stability During Cation Exchange Chromatography. (Manuscript Under Preparation)."

Chapter Four contains a detailed discussion of monoclonal antibody charge variants and how they can be identified via membrane chromatography.

- "R. Sadavarte, P. Madadkar, C.D.M. Filipe, R. Ghosh. Rapid Preparative Separation of Monoclonal Antibody Charge Variants Using Laterally-Fed Membrane Chromatography. J. Chromatogr. B. 1073 (2018) 27-33."

In **Chapter Five**, the advantages and disadvantages of the techniques presented in Chapters 2, 3, and 4 are considered, and recommendations are made for future work.

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CHAPTER 2: RECOVERY OF FUNCTIONALLY-ACTIVE PROTEIN FROM INCLUSION BODY USING A THERMAL-CYCLING METHOD

2.1. Abstract

Heterologous overexpression of genes in *Escherichia coli* has made it possible to obtain high titers of recombinant proteins. However, this can result in the formation of aggregated protein particles known as 'inclusion bodies'. Protein sequestered as inclusion body is inactive and needs to be converted back to its functional form by refolding using appropriate techniques. In the current study inclusion bodies of the enzyme aminoglycoside nucleotidyl transferase (or ANT(2")-Ia) were first solubilized in urea and subsequently subjected to thermal-cycling under controlled conditions as part of the refolding strategy. Thermal-cycling led to disaggregation of the individual protein chains and simultaneously refolding the released protein molecules to their native state. The optimum condition was identified as 10-80°C thermal-cycling at 3°C/sec for 2 h. Enzyme activity measurements showed that thermal-cycling under optimized conditions resulted in 257% activity recovery when compared with non-refolded protein.

Keywords: inclusion body, thermal cycling, disaggregation, protein refolding, enzyme activity

2.2. Introduction

Protein over-expression in genetically modified host organisms is routinely performed to obtain higher product titers.[1,2] Such over-expression typically leads to the formation of non-native protein aggregates referred to as inclusion bodies (IBs), which can be distinguished from soluble protein molecule as being bulky and non-functional.[3,4] In

theory, all proteins can form aggregates when exposed to specific stressed conditions.[5] Aggregates are held together by different intra- and intermolecular interactions. In bioprocessing, protein aggregates are generally undesired impurities which could potentially induce toxic or immunogenic responses.[6-8] Removal of protein aggregates from biopharmaceutical products is technically difficult and increases the manufacturing cost. However, formation of IBs by recombinant proteins can also be viewed as advantageous from a bioprocessing point of view because it increases protein yield, reduces protein degradation by hydrolysis, and facilitates protein separation and recovery.[4,9]

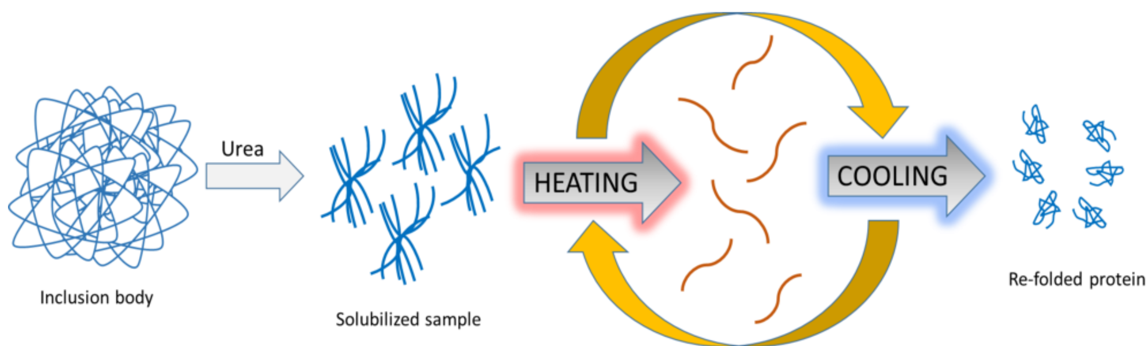

Figure 2.1: Working hypothesis for refolding of inclusion body derived protein by thermal-cycling.

Several studies have shown IBs are not completely inactive but show some residual activity.[10] It has also been argued that protein precipitation or inclusion body formation could be linked to thermodynamic stabilization.[11] This has been verified by comparison of soluble cytoplasmic proteins with their IB counterparts.[10] Inclusion body proteins need to be appropriately processed to obtain stable, properly refolded functional protein molecules

before they can be used for their intended biopharmaceutical applications.[12,13] In most protein refolding protocols, IB aggregates are first exposed to high concentrations of denaturants like guanidine hydrochloride (6 M) or urea (8 M).[14] These denaturants most likely disrupt intramolecular hydrogen bonds in the constituent protein molecules, thereby solubilizing the protein which exist as individual uncoiled molecules.[15,16]

The next step in most refolding strategies involve controlled removal of denaturant to refold the solubilized protein molecules back to their native structure accompanied by restoration of functionality.[17-20] The composition of the refolding buffer is specific to the protein being processed. Chemical species present in such a buffer provide oxidizing/reducing conditions which improve refolding yield. Since the refolding behavior is equilibrium controlled, it proceeds extremely slowly.[17,21-23] Co-solutes[24] or molecular chaperones[25] have been used to improve refolding yield. Non-chemical refolding methods involving the use of high pressures have been reported but these need highly specialized equipment to perform experiments and are expensive for operation.[26,27]

An alternative approach for protein refolding based on thermal-cycling was recently reported.[28] This method was successfully used for disaggregation of non-native monoclonal antibody oligomers to obtain refolded and biologically active monomeric antibody molecules. The method involved exposure of protein sample to multiple heating and cooling cycles with minimal temperature holding. In the current work we have used the enzyme aminoglycoside nucleotidyl transferase (2”) – Ia [ANT(2”) – Ia], also known as gentamicin 2”-nucleotidyltransferase (EC 2.7.7.46), as a model inclusion body forming

protein. ANT(2")-Ia is naturally produced by bacteria as self-defense against antibiotics and has been studied over the past 2-3 decades for its ability to catalyze the chemical

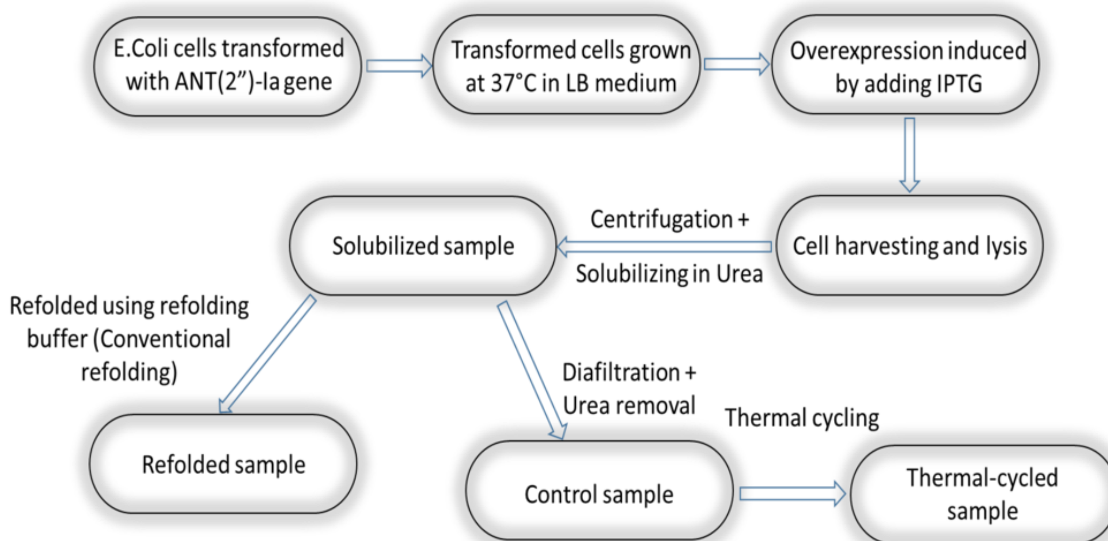

Figure 2.2: Schematic diagram showing various steps involved in ANT(2")-Ia extraction, purification and refolding.

reaction of converting potent antibiotic molecules into inactive state by N-acetylation reaction pathway thereby decreasing their ability to attack the ribosomal RNA subunit in bacterial cells.[29-31] As the name suggests, this enzyme acts like a "transfer agent" and transfers a nucleotidyl moiety from an ATP molecule onto the active aminoglycoside antibiotic molecule. The resulting molecule becomes ineffective in killing the bacterial cell. This overall mechanism is shown below:

MgATP + 2"-hydroxyaminoglycoside ⟶ MgPPi + AMP-2"-aminoglycoside

ANT(2")-Ia is naturally produced by bacteria as a response to antibiotic attack and is therefore available only in minute quantities inside the cell. Large scale production of this enzyme is possible only by over-expression as an IB. IBs formed by heterologous overexpression of ANT(2")-Ia in *E. coli* was used as starting material in our study. Based on our earlier study[28] we hypothesized that it should be possible to obtain disaggregated, biologically active and properly refolded enzyme molecules by thermal-cycling under precisely optimized conditions. The proposed mechanism of protein disaggregation and refolding is shown in **Figure 2.1**. Disaggregation of ANT(2")-Ia was confirmed from dynamic light scattering (DLS), recovery of biological activity was verified using enzyme assay against kanamycin substrate, and refolding/structural changes were monitored by circular dichroism (CD) spectroscopy.

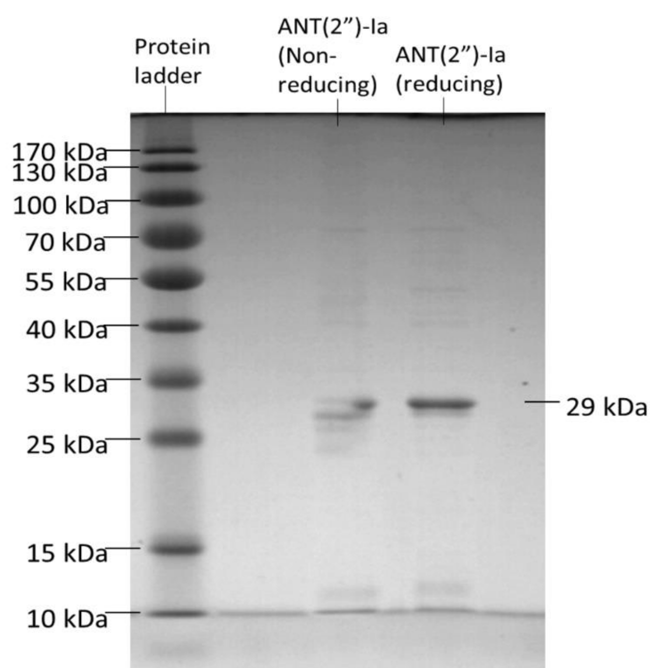

Figure 2.3: Reducing and non-reducing SDS-PAGE obtained with purified ANT(2")-Ia.

2.3. Experimental

2.3.1. Materials

E. coli BL21 (DE3) cells containing vector pET22b(+) and antibiotic resistance gene (ampR) and encoding the gene for ANT(2")-Ia were used as expression host. These were kindly provided by Dr. Gerry Wright, McMaster University. This protein contains 3 disulfide bonds in the backbone chain in its native state. Adenosine 5'-triphosphate disodium salt (A2383), ampicillin anhydrous crystalline (A9393), ammonium molybdate (A1343), glycerol (G2025), hydrochloric acid 37% (258148), isopropyl-β-D-thiogalactopyranoside (IPTG) (I6758), kanamycin disulfate salt (K1876), magnesium chloride anhydrous (M8266), malachite green carbinol hydrochloride (213020), peptone (P5905), potassium chloride (P9541), recombinant inorganic pyrophosphatase from E. coli (I5907), Triton X-100 (T8787), Trizma hydrochloride (T5941), and yeast extract (Y1000) were purchased from Sigma Aldrich, St. Louis, MO, USA. Sodium chloride (SOD 002.205) was purchased from Bioshop, Burlington, ON, Canada. Urea (17-1319-01) was purchased from GE Healthcare, Piscataway, NJ, USA. EDTA disodium salt dehydrate (37560) was obtained from Anachemia Chemicals Inc, Rouses Point, NY, USA and dithiothreitol (3870) was obtained from EMD Millipore, Gibbstown, NJ, USA. Purified water (18.2 MΩ cm) used in purifying IB and in preparation of buffer solution was obtained from a Diamond™ NANOpure (Barnstead, Dubuque, IA, USA) water purification unit. Genuine Axygen Quality PCR tubes (PCR-02-C; 20 µL thin wall) used for thermal-cycling experiments were purchased from Axygen Inc., CA, USA.

2.3.2. Bacterial culture and IB purification

E. coli cells were cultured overnight at 37°C in LB nutrient medium containing yeast, peptone and sodium chloride (1:2:2 weight ratio respectively) in shaker flasks (10 mL per flask) in the presence of 50 μg/mL ampicillin for selecting the transformed cells.[32] The overnight culture was transferred to 1 L of ampicillin containing LB medium and the cells were grown under conditions specified above. A schematic diagram showing the various steps involved in obtaining and processing IBs is shown in **Figure 2.2**. Cells were lysed using intermittent ultrasound in an ice bath for one hour to release the IBs. These IBs were solubilized in 0.1 M Tris HCl buffer (pH 8.0) containing 10 mM DTT and 8 M urea. After one hour the insoluble mass was removed by ultracentrifugation at 30,000g for 30 minutes. Two distinct reference protein samples were prepared after urea solubilization. The solubilized sample was dialyzed against 20 mM sodium phosphate buffer, pH 7.0 to obtain the "control sample". The solubilized sample was refolded using protocol reported in the literature,[32] followed by dialysis to obtain the "refolded sample". Samples obtained by thermal cycling were compared with both reference samples described above.

2.3.3. Conventional protein refolding procedure

IB of ANT(2") solubilized in 8 M urea was refolded according to the procedure by Wright and Serpersu.[32] Briefly, known quantity of IB was added to the refolding buffer [0.1 M Tris HCl pH 8.5 containing 0.2 M KCl, 0.4 M Arginine and 5 mM Glutathione (reduced)] and allowed to refold for 8 hours after which the enzyme was concentrated and simultaneously buffer-exchanged using 3K Amicon Ultra-15 Centrifugal Filter Units

(Millipore). The concentration step was performed three times. 20 mM sodium phosphate buffer (pH 7.0) was used during the above steps.

2.3.4. Refolding by thermal-cycling

Thermal-cycling experiments were performed using a PCR thermal-cycler (Eppendorf Mastercycler® personal; Applied Biosystems, Carlsbad, CA), using 200 µL PCR tubes with 20 µL capacity. Concentration of ANT(2")-la during thermal-cycling experiments was adjusted to give a final concentration 0.5 µM during the assay. Samples obtained after thermal-cycling were allowed to equilibrate to room temperature before further processing.

2.3.5. Reducing and non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

ANT(2")-Ia samples were dialyzed against 20 mM sodium phosphate buffer (pH 7.0) using Amicon Ultracel-3 membrane filter prior to analysis by 10% SDS-PAGE[33] using a Hoefer MiniVE system (GE Healthcare Biosciences, QC, Canada. The gels were stained using Coomassie Brilliant Blue dye. Images for the destained gel presented in this paper were obtained using a digital camera.

2.3.6. Circular Dichroism

Circular dichroism spectroscopy helps to monitor changes in protein structure. Proteins that undergo structural changes during refolding show distinct characteristics in the far-UV or near-UV profiles. In order to monitor the effect of thermal-cycling on secondary structure of ANT(2")-Ia, circular dichroism spectroscopy was carried out in the far-UV

wavelength (190-260 nm) region. Experiments were performed using a Model No. 416 CD spectrometer (AVIV Biomedical Inc., Lakewood, New Jersey). ANT(2”) samples (0.3 mg/mL) were added to a quartz cuvette having 1 mm path length. The cuvette was then placed in the instrument and scanned at a rate of 1 nm per 3 seconds with constant nitrogen purging at 25°C. Three measurements were obtained for each sample and the average value was reported. Data obtained was processed and reported in terms of mean residue ellipticity expressed in deg cm^2 $dmol^{-1}$.

2.3.7. Particle size analysis

Dynamic Light Scattering (DLS) measurements were performed using a Zetasizer Nano ZS instrument (Malvern Instruments Ltd, Worcestershire, UK). Protein samples were placed in 1 cm path length quartz cuvettes and exposed to 633 nm laser. The instrument incorporated non-invasive backscattering (NIBS) optics and therefore the intensity of scattered light was measured at an angle of 173° using avalanche photodiode. Each measurement was recorded as the average of 15 data acquisitions and 3 such measurements were made for each sample. Average particle size and corresponding polydispersity index values are reported. It is to be noted that the reported polydispersity index values are a qualitative measure of the average particle size distribution of different moieties present within the sample analyzed. This has also been concluded from SEC result for refolded ANT(2”)-Ia by Wright and Serpersu as being primarily monomeric.[32]

2.3.8. Enzyme activity assay

Enzyme activity was measured according to reported protocol[32,34] with slight modification. The reaction mixture consisted of 20 mM Tris (pH 8.0), 5 mM $MgCl_2$, 50 mM KCl, 10 mM DTT, 2.5 mM ATP and 250 µM kanamycin (substrate). Inorganic pyrophosphatase (2 units) was also included in the assay to ensure complete conversion of pyrophosphate to phosphate. Prior to activity measurement, 1 mL of reaction mixture was warmed to 37°C. ANT(2")-Ia was added to obtain a final concentration of about 0.5 µM. 40 µL of the solution was pipetted every minute into a developing solution. The developing solution was 3:1 parts by volume of 0.045% Malachite Green HCl (prepared in water) and 4.2% ammonium molybdate (prepared in 4 M HCl). The resulting solution was then incubated for 5 minutes at room temperature, followed by optical density measurement at 660 nm using a spectrophotometer. One unit of enzyme activity corresponded to the production of 1 µmol of phosphate per min. Specific enzyme activity was reported as units of enzyme activity per mg of ANT(2")-Ia present.

2.4. Results and Discussion

The "control sample" was first analyzed using SDS-PAGE under reducing and non-reducing conditions (see **Figure 2.3**). ANT(2")-Ia is a monomeric protein with molecular weight of approximately 29 kDa.[32] SDS-PAGE results indicate the presence of one major protein around 29 kDa range and several other impurities at relatively low concentrations, with high-molecular weight impurities accounting for < 10% of total protein (as computed

based on band intensity on gel). Since the "control sample" did not contain significant amounts of impurities, it was subjected to thermal-cycling without further purification. During the thermal-cycling process, a sample experiences very rapid changes in temperature. Overall, repeated heating and cooling results in disaggregation of non-native aggregates and simultaneous refolding and renaturation, as has been demonstrated with IgG1 monoclonal antibodies.[28] The heating phase of the cycle partially unfolds the temperature-sensitive protein domains by disrupting the non-covalent intra- and inter-molecular interactions. The rapid cooling phase that follows allows the unfolded protein chains to reorganize and refold into their native state.

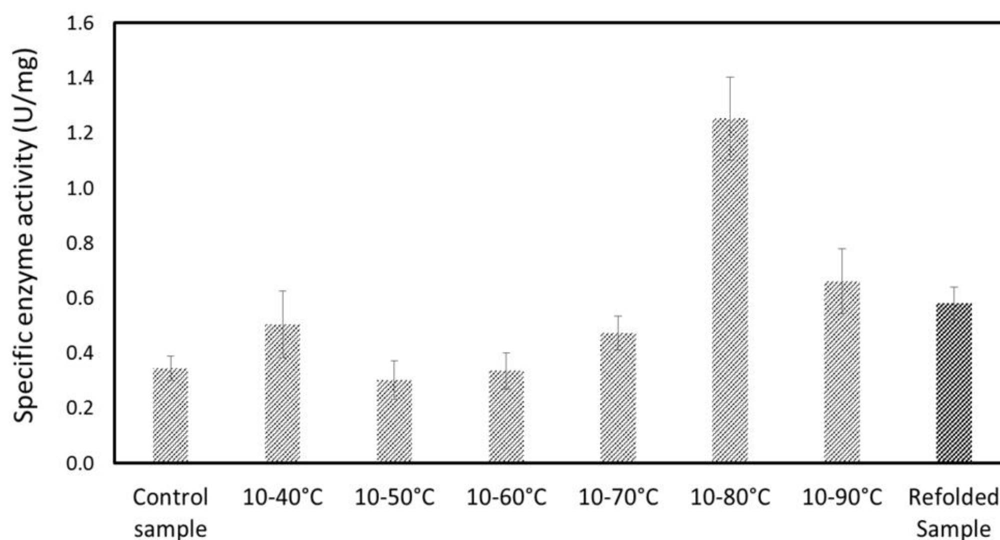

Figure 2.4: Effect of upper and lower temperature range on enzyme activity recovery by thermal cycling. All these experiments were carried out for 2 hours at 3°C/sec heating/cooling rates.

Different thermal-cycling protocols were tested to optimize conditions for maximum enzyme activity recovery. The initial thermal-cycling conditions were chosen based on the results of our previous monoclonal antibody disaggregation work.[28] The lower temperature was arbitrarily set to 10°C while the upper temperature was varied from 40°C, with increments of 10°C for each subsequent experiment.

The initial thermal-cycling experiments were carried out for 2 hours with the total number of heating and cooling cycles being forty-eight. The fastest heating and cooling rate used in the above experiments was therefore 3°C/sec. The results obtained are summarized in **Figure 2.4.** The data obtained with the "control sample" and the "refolded sample" is also shown for comparison. The "control sample" which showed a specific enzyme activity of 0.35 U/mg was prepared by removing urea for solubilized IB by centrifugal ultrafiltration. Since the rate of urea removal cannot be precisely controlled, the process used to generate the "control sample" represents a less than ideal refolding protocol. The proportion of properly refolded protein in resultant product is therefore expected to be low with a significant proportion of protein being present as random aggregates.

The conventionally "refolded sample" showed a significantly higher specific enzyme activity (i.e., 0.58 U/mg). Samples refolded by thermal-cycling showed a wide range of enzyme activity depending on the processing conditions. Based on data shown in **Figure 2.4**, it may be concluded that an upper temperature below 80°C did not result in significant activity recovery, when compared with "control sample". Thermal-cycling between 10-80°C gave the best specific enzyme activity (i.e., 1.25 U/mg). However, when the upper bound temperature was increased from 80°C to 90°C, there was a very significant drop in

the activity, presumably due to denaturation caused by unfolding and subsequent misfolding of the protein. Overall, the results suggest that ANT(2")-la unfolds at a temperature between 70 - 80°C. However, since the protein was not very pure, it was not possible to verify this using differential scanning calorimetry (DSC).

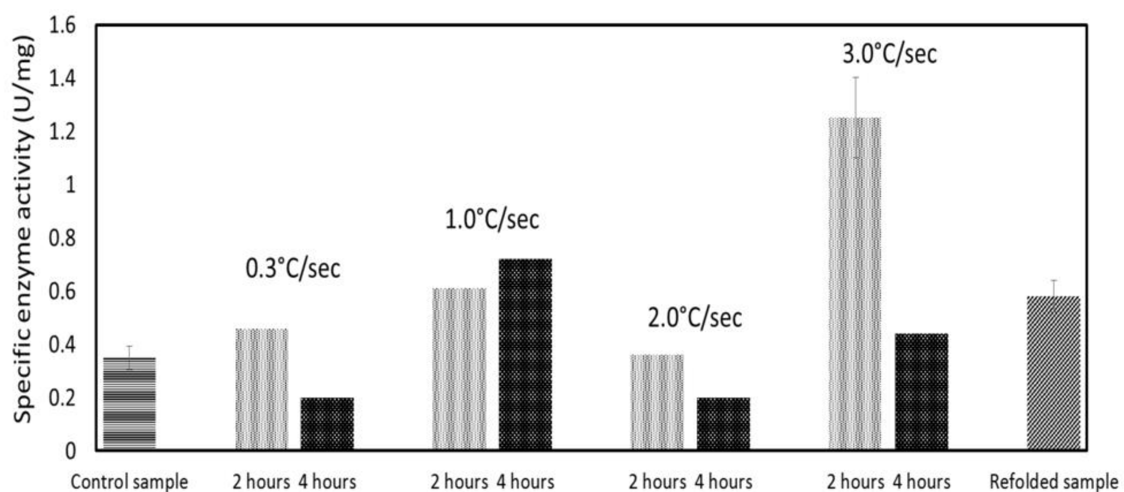

Figure 2.5: Effect of heating and cooling rates and time on enzyme activity recovery by thermal cycling. Upper and lower temperature range in all these experiments was 10-80°C.

Figure 2.5 summarizes the effects of the duration and the rates of heating/cooling in thermal-cycling experiments carried out using 10-80°C cycles. The highest recovery in enzyme activity was observed in a 2-hour experiment carried out using 3°C/sec heating/cooling rates. This is consistent with results obtained in our earlier study on monoclonal antibody disaggregation and refolding.[28] Quite clearly, the detrimental effects

of high temperature become more significant in experiments carried out for longer duration. However, no clear conclusion could be drawn on the effect of heating/cooling rates on activity recovery, other than 3°C/sec was the best condition in the experimental range examined.

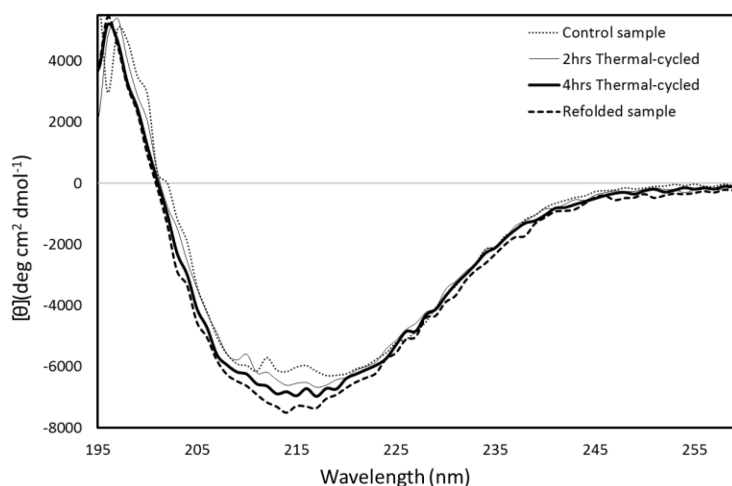

Figure 2.6: CD spectra obtained with "control sample", "refolded sample", and 2- and 4-hour thermal-cycled samples (10-80°C at 3°C/sec).

The effects of thermal stresses accompanying rapid temperature changes on the secondary structure of proteins have been examined by several researchers.[35,36] Whether such effects were significant in the above thermal-cycling experiments with ANT(2")-Ia was examined by circular dichroism (CD) spectroscopy. **Figure 2.6** shows the CD spectra obtained with samples thermal-cycled for 2 hours and 4 hours along with the "control sample" and the conventionally "refolded sample". All the samples showed negative peaks around 218 nm which indicates the presence of β-sheet secondary structure in proteins. The broadness of

these negative peaks indicates relaxed β sheet structure. Also, the fact that all four profiles are so similar suggests that the β-sheet composition does not change significantly after refolding.

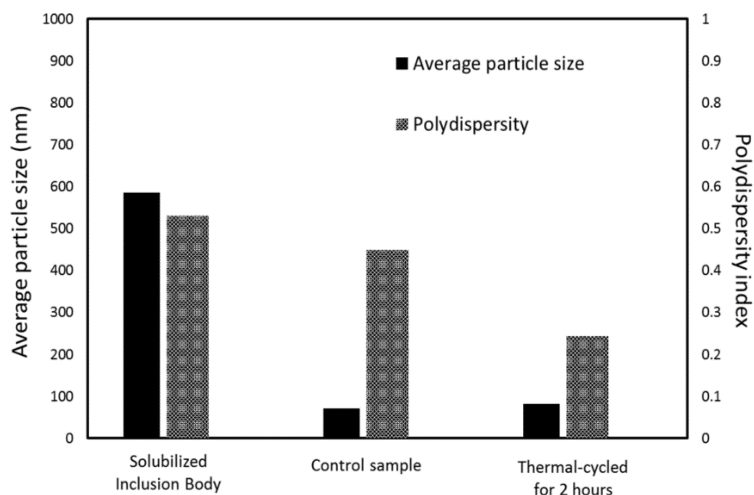

Figure 2.7: Particle size and polydispersity index data obtained with solubilized IB, "control sample" and thermal-cycled sample (10-80°C at 3°C/sec for 2 hours).

Refolding of ANT(2")-la was also assessed using dynamic light scattering (DLS) spectroscopy. **Figure 2.7** shows the particle size and polydispersity index data obtained with ANT(2")-la IB solubilized in 8 M urea, the control sample and refolded ANT(2")-la obtained by thermal cycling between 10-80°C for 2 hours at 3°C/sec heating/cooling rates. Protein solubilized in 8 M urea is expected to assuming a fully extended (uncoiled) configuration, which was confirmed by a larger particle size (585 nm) and a higher polydispersity index. On removal of urea, the protein would assume a folded configuration and therefore the "control sample" indicated significant reduction in particle size.

However, due to the randomness of folding and the resultant formation of differently folded species, the “control sample” showed a high polydispersity index. Further, when the “control sample” was thermal-cycled (10-80°C, 2 hours) the particle size remained almost unchanged but there was significant reduction in polydispersity, indicating a higher degree of homogeneity of refolded species.

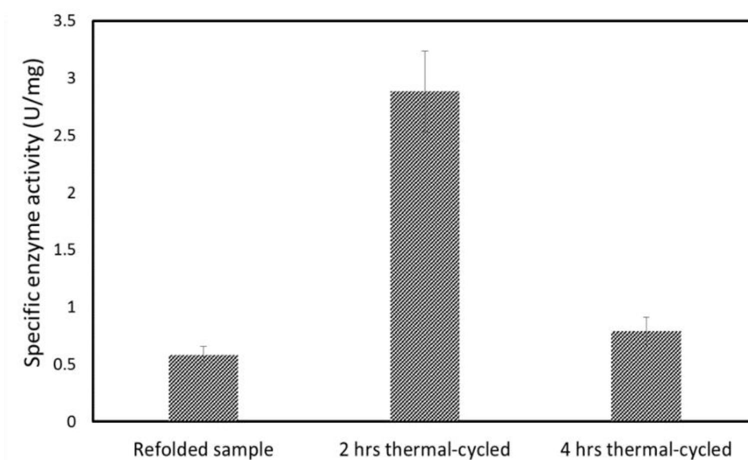

Figure 2.8: ANT(2”)-Ia activity obtained with “refolded sample” and those obtained by thermal-cycling it for 2 and 4 hours (10-80°C at 3°C/sec).

As discussed above, thermal-cycling of the “control sample” resulted in considerable increase in specific enzyme activity recovery (i.e., 257%). As a next step to testing the efficacy of the thermal-cycling method, the conventionally “refolded sample” was subjected to thermal-cycling between 10-80°C at 3°C/sec heating/cooling rates for 2 and 4 hours respectively, followed by measurement of enzyme activity. **Figure 2.8** summarizes the results obtained from these experiments. Thermal-cycling of the “refolded sample”

resulted in a 382% enhancement in specific enzyme activity. However, thermal-cycling for 4 hours resulted in only 35% increase in activity. Consistent with earlier experiments, thermal-cycling for extended duration lead to detrimental effects such as protein denaturation which contributed to a modest next enhancement in enzyme activity. **Figure 2.9** shows the particle size and polydispersity index data for conventionally "refolded" ANT(2")-la sample and those obtained by thermal cycling of the "refolded sample" between 10-80°C for 2 and 4 hours respectively at 3°C/sec heating/cooling rates. Thermal-cycling did not result in very appreciable changes to particle size. However, the polydispersity index values decreased considerably.

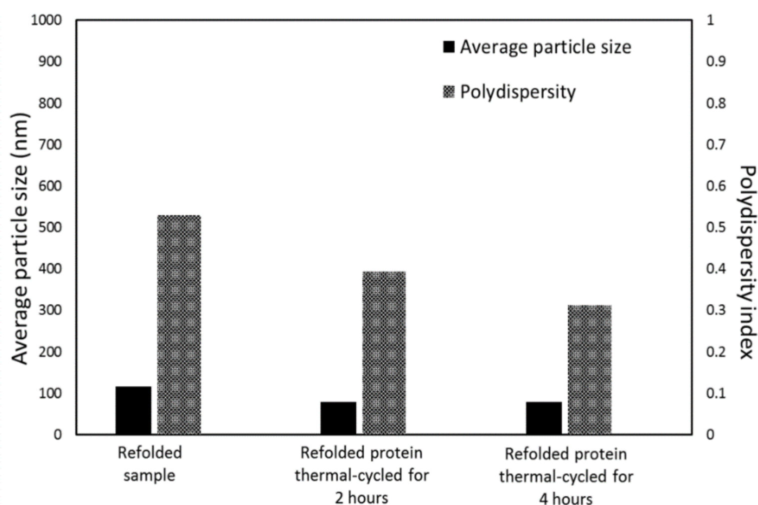

Figure 2.9: Particle size and polydispersity index data obtained with "refolded sample" and those obtained by thermal-cycling it for 2 and 4 hours (10-80°C at 3°C/sec).

2.5. Conclusion

ANT(2")-Ia inclusion body derived material could be refolded into functionally active protein using thermal-cycling. Specific processing conditions such as upper temperature, heating and cooling rates, and duration had significant effect on the efficacy of thermal cycling. An upper temperature below 80°C proved to be ineffective while there was significant drop in the activity at 90°C. Optimum activity recovery was observed when the protein sample was thermal-cycled between 10-80°C.

While no clear trend was noticed on the effect of heating/cooling rates on activity recovery, 3°C/sec was found the best condition in the experimental range examined. The reduction in enzyme activity with increase in duration of thermal-cycling from 2 to 4 hours show that detrimental effects of high temperature become more significant in experiments carried out for longer duration. Thermal-cycling, of the "control sample" for 2 hours between 10-80°C at 3°C/sec heating/cooling rates resulted in 257% enzyme activity recovery. Using a combination of conventional refolding and thermal-cycling, the activity recovery could be further increased to 382%. This indicated that the conventionally refolded protein was not fully active with further room for activity recovery. CD spectroscopy showed that ANT(2")-Ia predominantly consisted of β-sheet-like structure and thermal-cycling did not have any detrimental effect on its structure. DLS data further confirmed that ANT(2")-Ia could be refolded by thermal-cycling.

2.6. Acknowledgements

We like to thank Prof. Gerard Wright at McMaster University for generously donating the transformed *E. coli* cells used in this study. We also acknowledge help from Kun Zhang (Department of Biochemistry and Biomedical Sciences, McMaster University) for helping with initial cell culturing work.

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CHAPTER 3: EFFECT OF ON-COLUMN CONTACT TIME ON PROTEIN STABILITY DURING CATION EXCHANGE CHROMATOGRAPHY

3.1. Abstract

We examine the effect on on-column contact time during cation-exchange chromatography on protein stability. Conalbumin (77 kDa) was used as model protein as charge variants of this protein are known to exit in commercially available samples. When conalbumin was eluted immediately after adsorption on a sulfopropyl (SP) column, i.e., by maintaining the contact time to a minimum, two peaks corresponding to two charge variants were observed. When the contact time was increased by delaying the elution, the ratio of the eluted peaks changed dramatically, even though the elution buffer and gradient were identical to that used for the minimum contact time elution. The extent of delayed elution also had a significant impact on the ratio. Similar experiments were carried out using a second model protein, hemoglobin, which is also known to exist as several charge variants. As with conalbumin, the ratio of eluted peaks changed, and the eluted peaks became broader with increase in on-column contact time. These results could be explained in terms of structural relaxation of the protein, and this was verified using dynamic light scattering (DLS) and circular dichroism (CD) spectroscopy. The findings of this paper are relevant to process scale purification of biopharmaceutical proteins since during such separation processes, proteins remain bound to columns for considerable lengths of time before they are eluted.

Keywords: chromatography; protein stability; protein-ligand interaction; on-column contact time; protein structure; biopharmaceuticals

3.2. Introduction

Change in physicochemical properties of proteins due to their adsorption on surfaces has been widely observed and reported.[1,2] Adsorption of proteins on the surfaces plays a key role in bioprocessing, particularly bioseparations. In the biopharmaceutical industry, adsorption on the surface of resin particles is the basis for protein purification using chromatography. Chromatography is a popular choice for protein purification in the biopharmaceutical industry due to its high-resolution capabilities which allows its use for isolation of specific target products from very complex feed streams. The availability of a large array of separation chemistries makes chromatography the apex separation technique for purification of biopharmaceutical proteins such as monoclonal antibodies.

Protein adsorption also plays a critical role in chromatography-based analytical methods such as high-performance liquid chromatography (HPLC), or immunological assays such as ELISA and Western blotting.[3] By contrast, adsorption of proteins is considered the undesirable and even detrimental factor in separation processes such microfiltration and ultrafiltration as it leads to membrane fouling.[4] Even with chromatography, which relies on adsorption as the principal separation mechanism, fouling during repeated use, due to irreversible adsorption of proteins could be a serious problem.

The structure of a protein, which is stabilized by complex and subtle intramolecular interactions is designed to exist in solutions or to remain attached to specific molecular entities such as antigens and receptors.[6-8] While in the solution, the stability of a protein is affected by subtle changes in pH, ionic strength of buffering species, temperature, and

additives.[9-11] During chromatography, the protein of interest is transferred from the solution phase on to the ligands located on the surface of the resin particles. Such attachment of a protein to the surfaces chromatographic media could be described as unnatural as this is not what a protein molecule was designed for by nature, and this could potentially stress the protein and affect its structure, stability and biological activity.

The above could be a cause for concern when the protein being purified is intended for use as a biopharmaceutical,[12-15] or as an analytical reagent,[16,17] or for conducting diagnostic tests.[18,19] Destabilization of a protein molecule could result in unfolding and eventually denaturation. Denaturation is typically a two-step process involving reversible unfolding due to disruption of the secondary, and in some case tertiary structure, eventually followed by irreversible change in structure.[20] Several studies have shown protein denaturation ultimately leads to formation of aggregates or inclusion bodies.[21-23]

The conformational changes that a protein molecule undergoes during adsorption to and subsequent desorption from chromatographic media could potentially impact their structure, and thereby their stability and biological activity. Mazzer, et al.[24] have shown that monoclonal antibody molecules could be perturbed during adsorption on resin particles during their purification by protein A affinity chromatography, and this could contribute in a significant way towards their aggregation. They hypothesized that conformational changes along with changes in the hydration layer around the protein during its adsorption and desorption resulted in exposure of regions of the protein involved in unfolding transitions, ultimately leading to aggregation.

Papachristodoulou, et al.[25] examined destabilization of monoclonal antibody molecules during protein A chromatography using *in situ* neutron beam scattering and showed among other things how buffer conditions could alter the structure of a protein in its adsorbed state. Poplewska, et al.[26] discussed the mechanism of monoclonal antibody unfolding during cation exchange chromatography and explained how this resulted in peak broadening, multi-peak elution and reduction in recovery. More recently, Stańczak, et al.[27] used high-throughput methods for detecting unfolding of monoclonal antibodies on cation exchange resins and discussed how the antibody destabilized upon adsorption due to strong binding, which made it more prone to aggregate formation and loss in recovery.

Bind-and-elute chromatography, which is widely used for purifying protein biopharmaceuticals[5,24,27] relies on the modulation of solution properties in the vicinity of the protein and the resin particle by pH and/or ionic strength adjustment to promote binding during adsorption and disrupt binding during elution. In resin-based chromatography, the transport of different molecular species including the proteins being separated as well as the ionic species being used to manipulate the interactions between the protein and the ligand is limited by diffusion. Hence, the separation process could be very slow. This problem could be further amplified in process-scale chromatographic separations, typically used for manufacturing biopharmaceutical products, involving columns that could be several to hundreds of litres in bed volume.[28] Large-scale chromatographic purification processes could take several hours to complete.[29,30] The primary hypothesis of our current study is that the extent of the detrimental effects of protein-ligand interactions described in the previous paragraph would correlate with the duration for which a protein is sequestered

within a column, i.e., the on-column contact time. **Table 3.1** lists proteins for whom charge variants have been identified and studied indicating that such charge variants are commonly observed in living organisms.

Table 3.1: List of various proteins displaying charge variants in different living organisms

Protein	Size	Function
S100A4[31]	12 kDa	Calcium-binding protein
Ziv-Aflibercept (ziv-AFL)[32]	115 kDa	Treatment of metastatic colorectal carcinoma
L2 B-lactamase[33]	< 30 kDa	Resistance against β-lactams
rHGH[34]	22.12 kDa	Growth and Metabolism
Monellin[35]	10.7 kDa	Sweet protein
Human Serum Albumin[36]	66.5 kDa	Plasma protein
Chicken Growth Hormone[37]	26.3 kDa	Regulation of lipolysis, stimulation of protein, etc
Supercharged GFP[38]	~ 27 kDa	Binds to negatively charged RNA and DNA molecules
Peripheral subunit binding domain[39]	5 kDa	Facilitate shuttling of prosthetic groups between different catalytic subunits
Recombinant Oleosin[40]	20 kDa	Structural protein found in plant cells
Ferritin[41]	480 kDa	Iron storage protein
Green Fluorescent Protein[42]	27 kDa	In situ temporal gene expression
Angiotensin 1 Converting Enzyme 2[43]	85.9 kDa	Helps control blood pressure in humans
Human estrogen receptor alpha (hERα)[44]	65-70 kDa	Regulates eukaryotic gene expression, affects cell proliferation and differentiation in target tissues
Interleukin 23 receptor (IL23R)[45]	71.72 kDa	Protection against Crohn's disease and ulcerative colitis
Cold shock B protein[46]	7.3 kDa	Multifunctional RNA/DNA binding protein

In this paper, we examine the effects of on-column contact time on protein stability during cation exchange chromatography. For this study, we selected two model proteins,

conalbumin and hemoglobin, both of which are known to exist as charge variants. The secondary hypothesis of this study was that any on-column effect on the stability of these proteins would be observable as change in the ratio of these variants. The two proteins were individually bound to a 1 mL HiTrap Sulfopropyl (SP) cation-exchange column for different lengths of time and subsequently eluted. Conalbumin was eluted using a salt gradient while in the case of hemoglobin, it was eluted by increasing the pH. The effect of on-column contact time on the ratio of charge variants eluted for each protein was systematically studied. Under appropriate experimental condition after protein adsorption to the column substrate, native and unfolded protein molecules are identified by delaying the onset of application of elution buffer gradient. Our hypotheses are validated by analyzing the eluted protein samples by dynamic light scattering (DLS) and circular dichroism (CD) spectroscopy. The results obtained are discussed.

3.3. Experimental

3.3.1 Materials

Conalbumin (C7786), hemoglobin (H7379), sodium acetate (S2889), sodium phosphate monobasic (S0751), and sodium phosphate dibasic (S0876) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glacial acetic acid (1000-1-91) was purchased from Caledon Laboratory Chemicals, Georgetown, ON, Canada. Sodium chloride (SOD 002.205) was purchased from Bioshop (Burlington, ON, Canada). The proteins were dissolved in appropriate buffers prepared using water (18.2 MΩ cm) obtained from a Diamond™ NANOpure (Barnstead, Dubuque, IA) water purification unit. HiTrap™ SP

HP (1 mL) ion-exchange column purchased from GE Healthcare Biosciences, Baie-d'Urfe, QC, Canada) and used as recommended.

3.3.2. Ion-exchange adsorption experiments

The effect of on-column contact time on protein stability was examined using a HiTrap™ SP HP (1 mL) ion-exchange column. The column contained porous Sepharose beads with average particle diameter of 34 µm and strong sulfopropyl (SP) cation exchange ligand. This chromatographic media is considered suitable for high performance, high-resolution protein purification. The chromatography experiments were carried out using an AKTA Prime Plus liquid chromatography system (GE Healthcare Biosciences, Baie-d'Urfe, QC, Canada).

All chromatography experiments were carried out at 4 mL/min flow rate. Conalbumin adsorption was carried out using 20 mM sodium acetate pH 5.0 buffer while hemoglobin adsorption was carried out using 20 mM sodium phosphate pH 5.5 buffer. In each case, the HiTrap™ SP HP column was equilibrated with the appropriate binding buffer, after which respective protein was injected. The proteins were held within the column for different durations by maintaining flow of the respective binding buffer. The on-column contact time of the proteins was varied by controlling the onset of the elution step. Conalbumin was eluted using a 40 mL linear salt gradient (elution buffer: 20 mM sodium acetate pH 5.0 buffer + 0.5 M NaCl). Hemoglobin was eluted by using a 5 mL pH gradient (elution buffer: 20 mM sodium phosphate, pH 7.5) Conalbumin fractions collected during elution were desalting and concentrated using Amicon® Ultra-4 centrifugal ultrafilters (Millipore,

Billerica, MA, USA) and analyzed using dynamic light scattering (DLS) and circular dichroism (CD) spectroscopy.

3.3.3. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Conalbumin feed sample was analysed by 10% SDS-PAGE as per the protocol reported by Laemmli.[47] Approximately 10-15 µg of protein was loaded on the gel, and electrophoresis was carried out with Tris-glycine running buffer (pH 8.3) at a constant voltage of 140 V using a Hoefer MiniVE system (GE Healthcare Bio-Sciences, Baie-d'Urfe, QC, Canada). The gel was stained with Coomassie Brilliant Blue dye for 15-20 minutes and destained using mixture of methanol, acetic acid and water. Photograph of the destained gel was obtained with a Sony Exmor (R DSC-WX7, Japan) digital camera.

3.3.4. Dynamic Light Scattering (DLS)

Dynamic Light Scattering (DLS) analysis was performed using a Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK) to study the effect of on-column contact time on the stability of conalbumin. Briefly, protein fractions collected during the chromatography experiments were transferred to disposable polystyrene cuvettes of 1 cm path length. The protein samples were exposed to 633 nm laser, and the intensity of scattered light was measured at an angle of 173° using avalanche photodiode, with the help of non-invasive backscattering (NIBS) optics. Each protein sample was analyzed in triplicate, and data obtained for each sample was computed based on the average of 15 data acquisitions. All measurements were performed at 23°C. From the information obtained, values for average protein particle size along with polydispersity index were determined.

It is to be noted that the polydispersity index only provides a qualitative measure of the variability in particle size within a protein sample.

3.3.5. Circular Dichroism (CD) Analysis

Protein unfolding causes structural disturbance that are reflected in secondary structure. The effect of con-column contact time on the secondary structure of conalbumin was assessed by Circular Dichroism (CD) spectroscopy. In CD, structural changes are determined by performing a wavelength scan and recording changes in ellipticity. Proteins absorb strongly in the near- and far-UV regions as is evident from their maximum absorption coefficient at 222 nm. Therefore, a far-UV scan was performed in the 190-260 nm wavelength range. Conalbumin samples collected during the chromatography experiments were adjusted to 0.3 mg/mL concentration and added to a quartz cuvette having 1 mm path length. During the wavelength scans from 260 nm to 190 nm, measurements were made at intervals of 1 nm wavelength. Three such scans were performed for each protein sample.

3.4. Results and Discussion

Figure 3.1 shows the chromatogram obtained when conalbumin bound on the HiTrap™ SP HP column and eluted immediately afterwards with the on-column contact time being very small. In this experiment the binding buffer was 20 mM sodium acetate (pH 5.0), while the eluting buffer was 20 mM sodium acetate (pH 5.0) 0.5 M NaCl. Commercially sourced conalbumin is known to contain charged variants.[48] The 40 mL linear gradient

used to elute conalbumin was selected to ensure that charge variants were indeed present, these would be eluted as separate peaks. provided better resolution and sharper peaks and was therefore chosen as the gradient length throughout this work. The chromatogram in **Figure 3.1** shows two distinct peaks labeled “peak 1” and “peak 2” respectively. In this experiment, the time difference between protein injection and initiation of the elution gradient was intentionally kept very small to ensure the on-column contact time could be kept low. Therefore, the ratio of the two charge variants in the eluted protein profile could be expected to be not too different from that in the feed sample.

From **Figure 3.1** it may be inferred that the “peak 2” variant bound more strongly to the sulfopropyl ligand that than “peak 1” variant. However, the presence of two peaks in a chromatogram obtained with a “pure” protein sample could also potentially imply presence of impurities. To verify whether the peaks seen in the chromatogram in **Figure 3.1** did indeed correspond to two conalbumin charge variants, the conalbumin feed sample was analyzed by 10% SDS-PAGE (see **Figure 3.2**). A single protein band corresponding to approximately 77 kDa molecular weight confirmed that the conalbumin sample used in our experiments was pure and that the two peaks seen in **Figure 3.1** were due to species of same molecular weight, albeit having slightly different charge. The presence of differently charged species in conalbumin has been reported in the literature.[48]

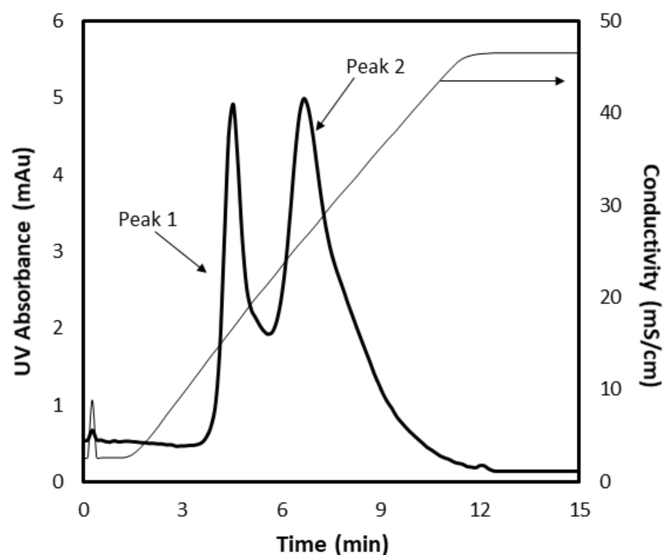

Figure 3.1: Chromatogram obtained during cation exchange chromatography of conalbumin on a HiTrap™ SP HP column with the minimum on-column contact time (protein concentration: 4 mg/mL; loop: 100 µL; flow rate: 4 mL/min; binding buffer: 20 mM sodium acetate, pH 5.0; eluting buffer: 20 mM sodium acetate, pH 5.0, + 0.5 M sodium chloride; gradient: 40 mL, linear; contact time: 10 seconds).

Next, the on-column contact time for conalbumin on the HiTrap™ SP HP was systematically increased. **Figure 3.3** shows the chromatogram obtained when the on-column contact time was increased by delaying the onset of the elution step by 5 minutes, while **Figure 3.4** shows the chromatogram obtained when the on-column contact time was further increased by delaying the onset of the elution step by 10 minutes. In every other respect, the experiment represented in **Figure 3.1** was similar to those represented in **Figures 3.3 & 3.4**, i.e., same feed sample, buffers, flow rate and gradient length. A

comparison of the chromatogram shown in **Figures 3.1**, **3.3 & 3.4** indicate two main differences. Firstly, the height and area of "peak 1" greatly diminished with increase in on-column contact time of conalbumin.

Secondly, a shoulder appeared at the tail end on "peak 2". Therefore, there was a clear change in the ratio of variants, and likely appearance of an additional variant (i.e., the component present in the shoulder). Also, the longer on-column contact time increased the "overall" strength of interaction between conalbumin and the sulfopropyl ligand. When the delay in elution was increased from 5 minutes (see **Figure 3.3**) to 10 minutes (see **Figure 3.4**), "peak 1" decreased to tiny shoulder, while shoulder on the tail end of "peak 2" increased further, almost approaching the crest of the peak. In all three chromatograms (i.e., in **Figures 3.1**, **3.3 & 3.4**) the total area under the curve remained fairly constant. Therefore, any increase in the areas of the latter peaks (or shoulder) took place at the expense of those earlier.

Goheen and Hilsenbeck[49] while carrying out high-performance ion-exchange chromatography (HPIEC) experiments using human plasma proteins observed that the recovery of proteins decreased with increase in residence time in the column. Such loss in protein recovery was attributed to unfolding due to strong protein-substrate interaction, leading to stronger, and possibly irreversible binding of the proteins on the column. Based on the results discussed in the previous paragraph and based on the observations by Goheen

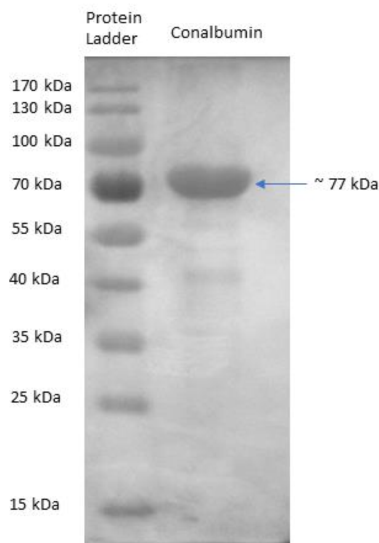

Figure 3.2: 10% SDS-PAGE analysis of conalbumin.

and Hilsenbeck,[48] our preliminary conclusion was that increased contact time of conalbumin on the HiTrap™ SP HP column increased the "strength" with which the protein bound to the ligand. This was probably due to structural changes in the protein which led to stronger interaction of the charged groups on the protein with that on the ligand. The change in the elution profile of conalbumin with increase in on-column contact time would also indicate that such structural change also led to change in charge variant composition of conalbumin. Whether such structural changes did indeed take place and whether these had any significant impact on the stability of conalbumin was examined using DLS and CD spectroscopy.

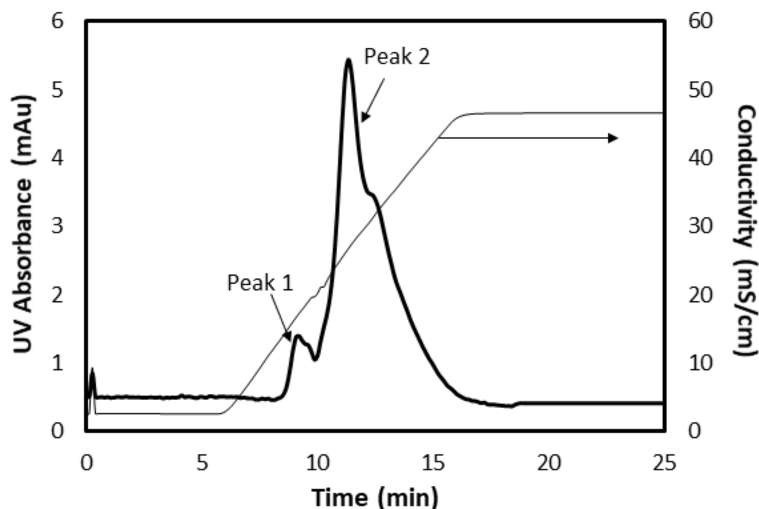

Figure 3.3: Chromatogram obtained during cation exchange chromatography of conalbumin on a HiTrap™ SP HP column with a 5-minute delay in the onset of elution (protein concentration: 4 mg/mL; loop: 100 µL; flow rate: 4 mL/min; binding buffer: 20 mM sodium acetate, pH 5.0; eluting buffer: 20 mM sodium acetate, pH 5.0, + 0.5 M sodium chloride; gradient: 40 mL, linear; contact time: 300 seconds).

A direct way to assess structural alterations in a protein to look for changes in physical properties of the molecule, such as its size. Therefore, samples collected from the individual peaks of the chromatograms shown in **Figures 3.1**, **3.3 & 3.4** were analyzed by DLS. The result obtained from these experiments are summarized in **Figure 3.5**. The conalbumin feed solution, i.e., conalbumin dissolved in 20 mM sodium acetate buffer (pH 5.0), was analyzed by DLS and its diameter was found to be 9.79 nm. The results obtained with the "peak 1" and "peak 2" samples obtained from the chromatography experiments

show some clear trends. Firstly, the conalbumin variant present in the second peak was larger than the one present in the first peak.

Secondly, the size of conalbumin in these peaks increased with increase in on-column contact time. For instance, the size of both variants obtained from the chromatography experiment with minimum on-column contact time (i.e., 10 seconds) was lower than conalbumin in the reference material (i.e., the feed). This slight discrepancy was probably

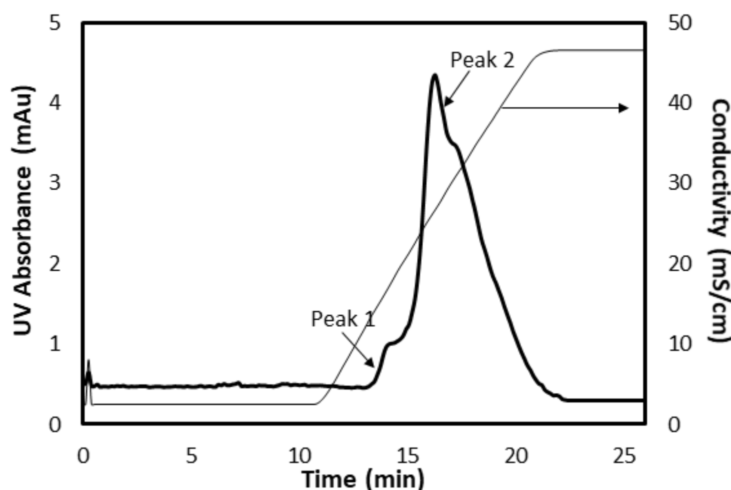

Figure 3.4: Chromatogram obtained during cation exchange chromatography of conalbumin on a HiTrap™ SP HP column with a 10-minute delay in the onset of elution (protein concentration: 4 mg/mL; loop: 100 µL; flow rate: 4 mL/min; binding buffer: 20 mM sodium acetate, pH 5.0; eluting buffer: 20 mM sodium acetate, pH 5.0, + 0.5 M sodium chloride; gradient: 40 mL, linear; contact time: 600 seconds).

due to the fact that the feed sample was a mixture of both variants, while the peak samples obtained from the chromatography experiment contained fractionated proteins. When the on-column contact time was increased to 300 seconds, the size of conalbumin

in both peaks increased very significantly. Further increase in on-column contact time increased the size of both peak fractions even further.

Protein unfolding, aggregation, denaturation or misfolding usually affect its secondary structure. With samples obtained from conalbumin chromatography experiments, the increase in molecular size indicates unfolding, which would affect both secondary and tertiary structure. Conalbumin shows a predominantly α-helix secondary structure, and any unfolding due to increased interaction with the sulfopropyl ligand on the cation exchange column would affect its α-helix content. **Figure 3.6** shows the CD spectra obtained with the different conalbumin samples. The conalbumin reference sample (protein dissolved in 20 mM acetate buffer, pH 5.0) gave two negative minima at 222 nm and 208 nm respectively. The eluted conalbumin samples used for obtaining the CD spectra consisted of both "peak 1 and "peak 2" species. This was done in order to objectively compare these with the reference conalbumin sample which contained both variants. A comparison of the three spectra in **Figure 3.6** shows increase in on-column contact time resulted in increase in the magnitude of residual ellipticity for conalbumin, which indicated structural disturbance due to unfolding.

In the conalbumin chromatography experiments discussed in the preceding section, elution was achieved by increasing salt concentration. Elution of proteins from ion-exchange columns can also be achieved by changing pH. In order to examine the effect of elution by pH change, experiments were carried out with the HiTrap™ SP HP column, using hemoglobin as model protein. In these experiments, 20 mM sodium phosphate (pH 5.5) was used as binding buffer and 20 mM sodium phosphate (pH 7.5) was used as eluting

buffer, the linear gradient length for elution being 5 mL. The results obtained from the hemoglobin chromatography experiments are summarized in **Figure 3.7**. In these experiments, the elution of hemoglobin was delayed by 5, 10 and 15 minutes respectively.

In each of these chromatography experiments, two eluted hemoglobin peaks were obtained. As with conalbumin, the on-column contact time had a profound effect on the eluted protein profile. A careful comparison of the three chromatograms shown in **Figure 3.7** indicates three trends. Firstly, increasing the contact time increased the area of the second peak at the expense of the area of the first peak. This trend was similar to that observed in the conalbumin chromatography experiments. The second trend in the hemoglobin chromatography experiments was that increase in on-column contact time resulted in significant broadening of the eluted protein peaks. The third trend was that the total peak areas of the hemoglobin peaks decreased slightly with increase in on-column contact time, which clearly indicated some loss in hemoglobin recovery from the column. The second and third trends observed in the hemoglobin chromatography experiments were not observed in the conalbumin chromatography experiments.

The usual suspects that could cause destabilization of proteins during ion-exchange chromatography are the strong protein-ligand electrostatic interactions, the dynamic changes in the ionic strength and the pH in the immediate vicinity of these interactions, and any additional interactions between the protein and the solid surface on which the ligands are attached. Other process related factors that could also play their role in this include temperature and additives. An additional factor that could be considered is protein concentration on the surface of the resin particles.

Studies carried out using hemoglobin have shown that protein adsorption on a surface could frequently be multi-layered, wherein the layer directly in contact with surface display rigid behavior and contain denatured protein, whereas the subsequent layers on top of this rigid layer were loosely attached.[50] Under pressure, as within a chromatography column, the loosely bound layer would undergo compaction and this would promote protein-protein interactions, leading to dimer and higher-order protein aggregates formation. The DLS results discussed in **Figure 3.5** indicate that the size of conalbumin in the eluate increased by as much as 80% relative to the reference. Therefore, prolonged sequestration of a protein in a column could quite possibly lead to the formation of higher ordered structures such as aggregates.

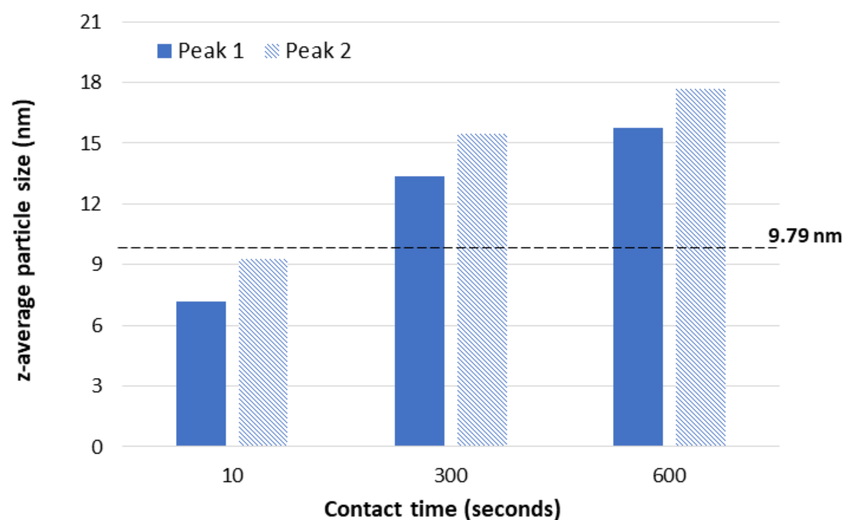

Figure 3.5: Effect of on-column contact time on conalbumin particle size in eluted samples collected during cation exchange chromatography (dashed line represents size of reference conalbumin).

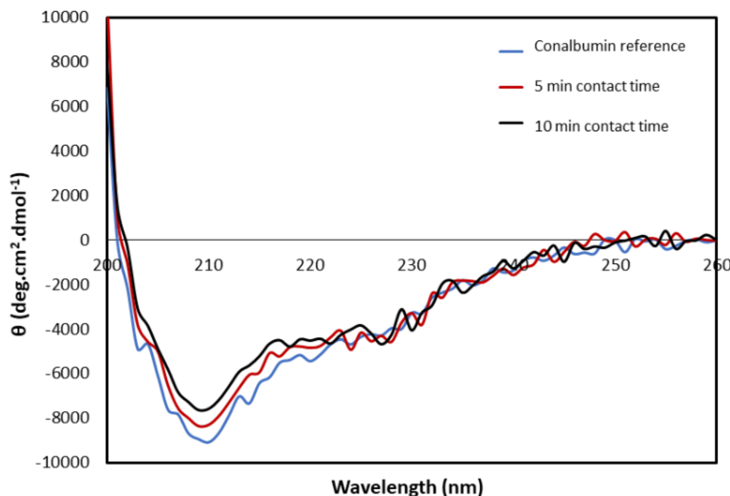

Figure 3.6: Far-UV circular dichroism (CD) spectroscopy analysis of conalbumin samples.

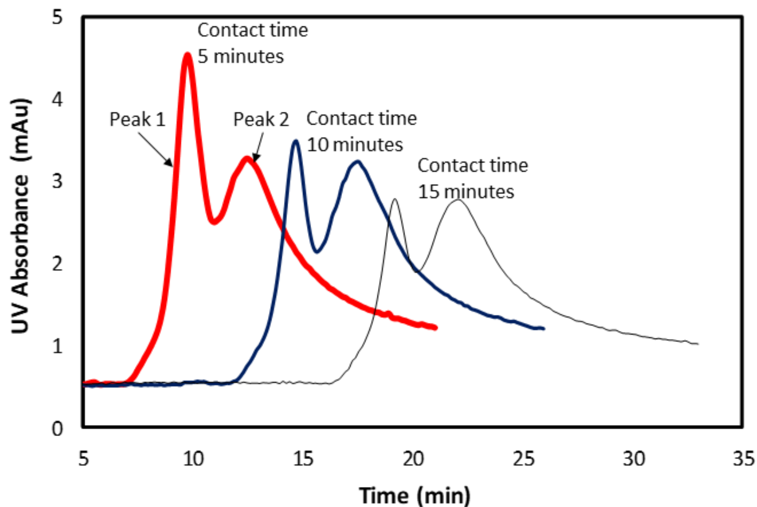

Figure 3.7: Chromatogram obtained during cation exchange chromatography of hemoglobin on a HiTrap™ SP HP column with different delays in the onset of elution (protein concentration: 4 mg/mL; loop: 100 µL; flow rate: 4 mL/min; binding buffer: 20 mM sodium phosphate, pH 5.5; eluting buffer: 20 mM sodium phosphate, pH 7.5; gradient: 5 mL, linear).

The results obtained with both conalbumin and hemoglobin clearly demonstrate the impact of on-column contact time on protein stability during cation exchange chromatography. It is well known that the binding of proteins on different types of solid surface can create stresses that could destabilize their structure.[1-3] Our study shows that the duration of such binding could have a very significant impact on the extent of such destabilization, i.e., the longer the duration, the greater the impact. Maintaining a protein in a bound state on a column for an extended duration of time, such as that used during large-scale manufacture of biopharmaceuticals would potentially have unintended consequences, and these need to be carefully evaluated. An indirect but very important conclusion of this study is that the detrimental effects on a protein could be minimized by decreasing the processing time, i.e., a faster separation would likely result in less damage. An obvious recommendation based on this conclusion is that predominantly convection based chromatographic separations such as membrane chromatography[51] would, in addition to being faster, and therefore being more productive, would also ensure greater stability of the biopharmaceuticals being purified.

3.5. Conclusion

The structure of proteins could be destabilized during chromatographic separations due to stresses generated due to interactions with the ligands. The results discussed in the paper show that with both conalbumin and hemoglobin, on-column contact time had a significant impact on protein stability during cation exchange chromatography. The extent of

destabilization increased with increase in on-column contact time. Both conalbumin and hemoglobin exist as charge variants, and the impact of on-column contact time on the stability of these proteins could be observed in terms of change the ratio of these variants.

With both proteins, increase in on-column contact time resulted in an increase in the proportion of the more strongly retained variant, at the expense of the less strongly retained variant. Increased on-column contact time of conalbumin resulted in structural changes in the protein which led to stronger interaction of the charges groups on the protein with that on the ligand. Such structural changes were verified by dynamic light scattering (DLS) and circular dichroism (CD) experiments. The size of the eluted conalbumin species was found to be bigger than the reference conalbumin molecule. Also, increase in on-column contact time of conalbumin resulted in increase in the magnitude of residual ellipticity, which indicated structural disturbance due to unfolding of protein. With hemoglobin, increase in on-column contact time resulted in significant broadening of the eluted peaks and some loss in hemoglobin recovery from the column.

Our study shows that allowing a protein to remain in a bound state on a column for an extended duration of time could potentially have unintended consequences. The findings of this study are particularly relevant to large-scale purification of biopharmaceuticals protein by chromatography, which is slow, and typically involves sequestration of these proteins within columns for significant lengths of time.

3.6. Acknowledgements

The authors would like to acknowledge support from the Natural Sciences and Engineering Research Council (NSERC) of Canada. We also thank Drs. Raquel and Richard Epand from the Department of Chemical Biology for access to CD spectroscopy instrument and the Biointerfaces Institute at McMaster University for help with DLS measurements.

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CHAPTER 4: RAPID PREPARATIVE SEPARATION OF MONOCLONAL ANTIBODY CHARGE VARIANTS USING LATERALLY-FED MEMBRANE CHROMATOGRAPHY

4.1. Abstract

Monoclonal antibodies undergo various forms of chemical transformation which have been shown to cause loss in efficacy and alteration in pharmacokinetic properties of these molecules. Such modified antibody molecules are known as variants. They also display physical properties such as charge that are different from intact antibody molecules. However, the difference in charge is very subtle and separation based on it is quite challenging. Charge variants are usually separated using ion-exchange column chromatography or isoelectric focusing. In this paper, we report a rapid and scalable method for fractionating monoclonal antibody charge variants, based on the use of cation exchange laterally-fed membrane chromatography (LFMC). Starting with a sample of monoclonal antibody hIgG1-CD4, three well-resolved fractions were obtained using either pH or salt gradient. These fractions were identified as acidic, neutral and basic variants. Each of these fractions contained intact heavy and light chains and so antibody fragmentation had no role in variant generation. The separation was comparable to that using column chromatography but was an order of magnitude faster.

Keywords: monoclonal antibody, charge variants, oxidation variants, separation, analysis, membrane chromatography

4.2. Introduction

Monoclonal antibodies (mAbs) belonging to the IgG1 subclass occupy a major portion of the therapeutic antibody market.[1,2] Most of the currently used IgG1 mAbs have almost identical Fc regions allowing their use for diverse applications by modulation of the variable Fab region.[2] Purification and polishing steps employed during mAb processing often destabilize the protein and in extreme situations leads to protein aggregation,[3–5] or chemical modifications like oxidation,[6,7] deamidation,[8] racemization,[9] and hydrolysis.[9] The final mAb formulation for therapeutic use is required to be substantially free of such degradation products as they tend to have lower biological activity, altered pharmacokinetic profiles, and in some case, these may induce severe immunogenic responses.[10] Therefore, mAb formulations often contain sugars and polyols,[11,12] polymers such as PEG,[13] surfactants,[14,15] amino acids,[16] and other additives to stabilize the complex structure of the protein.

According to International Conference on Harmonization (ICH) Q6B specifications, undesired physical and chemical changes to a monoclonal antibody throughout its lifetime are categorized as either process-related or product-related.[17] These guidelines recommend thorough characterization of mAb products to test for such changes. Impurities resulting due to physical degradation (aggregation, fragmentation, etc) are easier to detect. The more challenging task is to identify chemical modification to mAb molecules. Chemical modification usually does not affect the antibody backbone structure but alters properties such as hydrophobicity,[18,19] surface charge,[18] isoelectric point (pI)[20-26] and local conformation.[27,28] Since chemical modifications frequently produce changes in charge,

these modified molecules are often called charge variants. Various pathways leading to formation of monoclonal antibody charge variants have been comprehensively described.[19,29]

Stracke et al.[30] have reported significant differences in binding of oxidized and non-oxidized IgG1 mAb to FcRn receptors, i.e., the oxidized mAb bound weakly and was cleared faster, resulting in shorter half-life in mouse models. Boswell et al.[31] in their study reported differences in tissue distribution and consequently pharmacokinetic properties of mAb variants injected in rat model. The effect of antibody variants on serum half-life and clearance rate in mouse models has also been reported by Igawa et al.[32] It is now widely acknowledged that antibody variants affect the pharmacokinetic properties of a mAb drug and therefore any efficient method for separation and analysis of such variants would be extremely valuable tools for manufacturing these molecules.[30-32]

Presently, mAb variants are separated and analyzed by column chromatography or electrophoresis. Column-based methods typically include ion exchange chromatography (IEX),[33, 34] hydrophobic interaction chromatography (HIC)[35] and reverse phase-high performance liquid chromatography (RP-HPLC).[36] While these techniques give good resolution, separation time tends to be long. Moreover, the resin could be easily fouled and the need for frequent column cleaning increases the effective separation time even further. Electrophoretic methods include isoelectric focusing,[20,21] capillary electrophoresis[22–24] and 2D-gel electrophoresis.[25,26] These methods rely on the use of specific buffers with carefully chosen ionic strength and pH that allow subtle changes on a protein's surface to be detected. Even though these methods provide good resolution, they are cumbersome and time

consuming. Moreover, an electrophoretic method is tailored specifically for a particular protein and the operational parameters and strategies are not globally applicable.

Column chromatography is widely used for both analytical and preparative bioprocessing because of its directness and versatility. The main drawback of resin-based columns is that mass transfer is limited by diffusion which slows down the separation. By contrast, the mass transport in membrane chromatography,[37] which has been proposed as alternative to packed-bed chromatography, takes place predominantly by convection, which allows faster separation. Thus, separation time with membrane chromatography could be an order of magnitude lower than with equivalent column chromatography. However, up until recently, membrane chromatography was not considered suitable for high-resolution separations.

Using a new technique called laterally-fed membrane chromatography (LFMC) it is now possible to combine high-speed with high-resolution in membrane chromatography.[38-42] For instance, using this technique, it has been possible to perform fast analytical[41] and preparative[42] separation of monoclonal antibody aggregates. Our study on preparative separation of monoclonal antibody aggregates[42] demonstrated that sharp, well-resolved mAb monomer/aggregate peaks could be obtained at very high flow rates using a stack of cation exchange membranes as chromatographic media. In the present study, we use of an LFMC device based on the same membrane and a similar design to fractionate variants present in old samples of hIgG1-CD4, which is an IgG1 mAb.

As mentioned earlier, monoclonal antibodies of the IgG1 subclass undergo degradation through various pathways and which alters their properties. This converts a homogenous mAb sample into a non-homogenous variant-containing mixture. The separation strategy used in the current study was to bind non-homogenous mixture of hIgG1-CD4 molecules, irrespective of the extent of chemical modification, on a stack of cation exchange membrane in the presence of slightly acidic buffer and sequentially elute these out in order of increasing pI using either salt or pH gradient. Our results showed that fast, high-resolution separation of the different variants could be achieved using our LFMC devices. The results obtained are discussed.

4.3. Experimental

4.3.1. Materials

Purified mAb hIgG1-CD4 monomer (batch 12, 23rd March 1999) was kindly donated by the Therapeutic Antibody Centre (Oxford, United Kingdom). The monomer mAb as received was free from variants based on orthogonal chromatographic and electrophoretic testing[43] but prolonged storage and handling (e.g., freeze-thaw) of the material resulted in the formation of variants and some aggregates. Based on storage and handling logs, the number of freeze-thaw cycles was 15. Sodium phosphate monobasic (S0751) and sodium phosphate dibasic (S0876) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium chloride (SOD 002.205) was purchased from Bioshop (Burlington, ON, Canada). Buffers were prepared using purified water (18.2 MΩ cm) obtained from a Diamond™ NANOpure

(Barnstead, Dubuque, IA) water purification unit. Amicon® Ultra-4 Centrifugal Filters (fitted with Ultracel-50 membrane) purchased from Millipore (Billerica, MA) were used for buffer exchanging and concentrating mAb samples. For comparison of the results obtained using cation exchange membrane chromatography, control experiments were carried out using 1 mL analytical scale HiTrap™ SP FF and Hi Trap™ SP HP columns (GE, Mississauga, ON, Canada) which were used as per the manufacturer's recommendation.

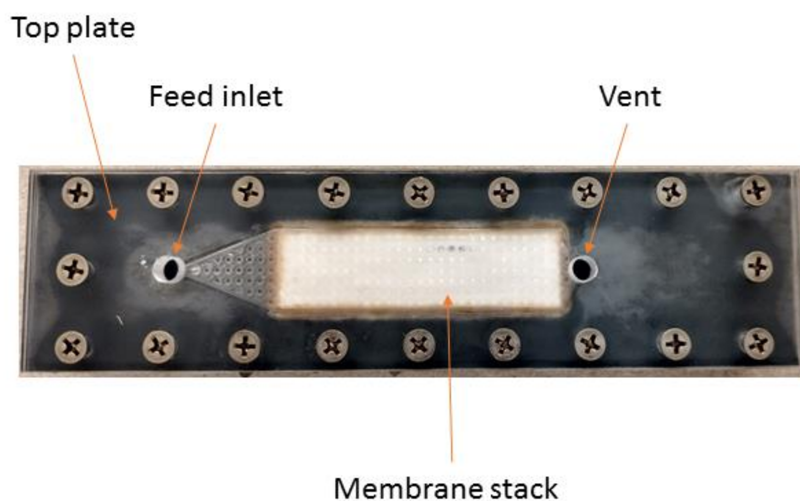

Figure 4.1: Top view of the 1 mL bed volume laterally-fed membrane chromatography (LFMC) device.

4.3.2. LFMC device

The LMFC devices used in this study were designed according to the details provided elsewhere.[42] A 1 mL bed volume device (see **Figure 4.1**) was used for analytical

separations while a 4.96 mL bed volume device was used for preparative separations. Briefly, these devices consisted of a top plate with an inlet port leading to the top channel of the device, and a bottom plate with an outlet leading out from the bottom channel, the inlet and outlet being located at opposite sides of the device. A frame which housed a rectangular stack of Sartobind® S membrane was sandwiched between the top and the bottom plates. The membrane bed height of the 1 mL LFMC device was 2.75 mm while the length and width of the membrane in the stack were 38 mm and 12 mm respectively. The corresponding dimensions of membrane stack in the 4.96 mL LFMC device were 3.3 mm, 70 mm and 20 mm respectively. The channels in the top and bottom plates were provided with pillars for supporting the membrane stack as well as for ensuring uniform liquid distribution in these channels.

4.3.3. Protocol for measuring the efficiency of chromatography devices

The number of theoretical plates in chromatography devices provides information on its efficiency. Theoretical plate measurements were performed for the 1 mL HiTrap™ column and the 1 mL LFMC device. The mobile phase consisted of 0.4 M NaCl solution and efficiency was measured at different flow rates using an AKTA prime plus chromatography system (GE Healthcare Biosciences, Baie-d'Urfe, QC, Canada). After equilibration with mobile phase, 10 µL of 0.8 M NaCl solution (i.e., 1% of bed volume) was injected into the device and the change in conductivity of the device effluent was monitored. The conductivity peaks generated was analyzed to determine the residence time (t_R) and the peak width at half height ($w_{1/2}$). The number of theoretical plates (N) was calculated using the following equation:

$$N = 5.545\left(\frac{t_R}{w_{1/2}}\right)^2$$

The number of theoretical plates per meter was obtained by dividing *N* with the respective resin/membrane bed height. The calculated values obtained for the 1 mL HiTrap™ SP FF and the 1 mL LFMC device are shown in **Table 4.1**.

Table 4.1: Comparison of the efficiency as measured in terms of theoretical plates of HiTrap™ SP FF and LFMC devices

Flow rate (mL/min)	HiTrap SP FF (1 mL column) (N/m)	HiTrap SP HP (1 mL column) (N/m)	LFMC (1 mL) (N/m)
1	2500	4800	---
2	2000	4300	16400
3	1750	3900	---
4	1650	3600	15200
6	---	---	13200

4.3.4. Cation exchange chromatography

An AKTA prime plus liquid chromatography system was used for carrying out the chromatography experiments. Analytical scale separation experiments (at 1 mL bed volume) with salt gradient elution were carried out using 20 mM sodium acetate (pH 5.0) as binding buffer with 0.5 M NaCl for elution. The pH gradient elution experiments were carried out using 50 mM sodium phosphate (pH 5.8) as binding buffer and 50 mM sodium phosphate (pH 8.0) as eluting buffer. Different linear gradients were examined in the above experiments. The concentration of mAb in the feed solution was 2.38 mg/mL unless mentioned otherwise.

4.3.5. Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples collected during the preparative chromatography experiments were concentrated and desalted using Amicon® Ultracel-50 centrifugal ultrafilter and analyzed by 10% SDS-PAGE under reducing and non-reducing conditions according to the method reported by Laemmli.[44] Both types of SDS-PAGE experiments were carried out at 140 V using a Hoefer MiniVE system (GE Healthcare Bio-Sciences, Baie-d'Urfe, QC, Canada) using Tris-glycine running buffer (pH 8.3). The gels thus obtained were stained using Coomassie Brilliant Blue dye for 15 min followed by destaining with a mixture of methanol, acetic acid and water. The gels were photographed with a digital camera (Sony Exmor R DSC-WX7, Japan) for presentation in figures.

4.4. Results and Discussion

Monoclonal antibodies of the IgG1 subclass readily form acidic and basic variants,[45] with very small differences in terms of their surface charge and effective pI. This makes their preparative separation quite challenging. Numerous papers describe separation of charge variants present in mAb samples using cation exchange chromatography.[46-50] For instance, charge variants could be separated using shallow pH gradients,[50] but this results in long separation times and diluted product fractions. We attempted to separate hIgG1-CD4 (pI > 8.0) variants with a 1 mL HiTrap™ SP FF column using a 150 mL pH gradient at a flow rate of 1 mL/min. The chromatogram thus obtained is shown in **Figure 4.2A**, where a single broad peak is seen eluting between 15 to 30 minutes.

After our failure to separate hIgG1-CD4 variants with a 1 mL HiTrap™ SP FF column using a pH gradient, we attempted to carry out the same separation using a more conventional salt (i.e., conductivity) gradient (see **Figure 4.2B**). It can be seen that the chromatogram obtained with both approaches were almost identical. In both cases a single elute peak is seen clearly indicating that the column was not suitable for fractionating variants present in hIgG1-CD4 sample. As can be seen in Table 1, the efficiency of the 1 mL HiTrap™ SP FF column is not very high and hence the poor resolution. The main difference between the two chromatograms was the presence of a significant flow-through peak in the pH gradient experiment (see **Figure 4.2A)**. The binding buffers used in the two experiments were different. While phosphate buffer at pH 5.8 was used in the pH gradient experiment, acetate buffer at pH 5.0 was used in salt gradient experiment.

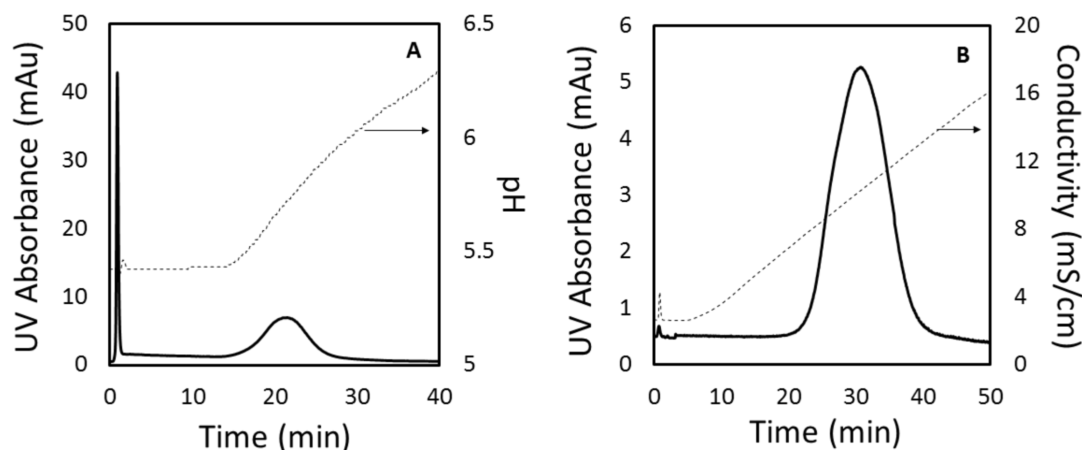

Figure 4.2: CEX analysis of hIgG1-CD4 using 1 mL HiTrap SP FF device using **(A)** pH gradient and **(B)** salt gradient (feed concentration: 2.38 mg/mL; loop: 100 µL; flow rate: 1 mL/min; gradient: 150 mL linear (0% - 100%); binding buffer for **A**: 50 mM sodium phosphate, pH 5.8; eluting buffer for **A**: 50 mM Sodium phosphate, pH 8.0; binding buffer for **B**: 20 mM sodium acetate, pH 5.0; eluting buffer for **B**: 20 mM sodium acetate, pH 5.0, + 0.5 M NaCl).

Although both pH values were lower than the pI of the mAb (pI > 8.0), a combination of buffer type and the lower pH ensured greater mAb binding in the salt gradient experiment. A 1 mL analytical HiTrap™ SP HP column was then used for fractionating variants present in the hIgG1-CD4 sample. The main difference between the HiTrap™ SP FF and the HiTrap™ SP HP columns is the resin particle size. While the former has an average particle size of 90 µm, the latter has an average particle size of 34 µm. Better separation was therefore expected with the HiTrap™ SP HP column. To test this hypothesis, hIgG1-CD4 fractionation experiments were carried out using the HiTrap™ SP HP column at two different flow rates i.e., 1 mL/min and 4 mL/min. The chromatograms obtained from these

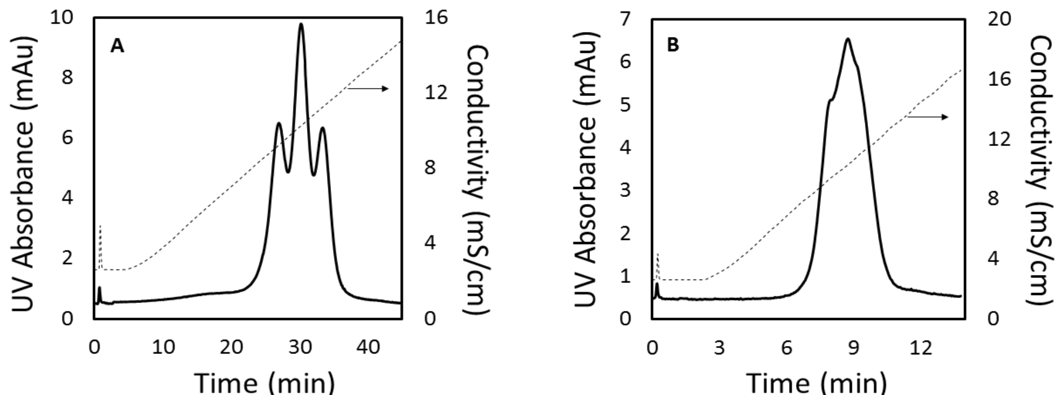

Figure 4.3: CEX analysis of hIgG1-CD4 with 1 mL HiTrap SP HP device using salt gradient at 1 mL/min flow rate **(A)** and 4 mL/min flow rate **(B)** (feed concentration: 2.38 mg/mL; loop: 100 μL; binding buffer: 20 mM sodium acetate, pH 5.0; eluting buffer: 20 mM sodium acetate, pH 5.0, + 0.5 M NaCl; gradient: 150 mL linear salt gradient, i.e., 0% - 100%).

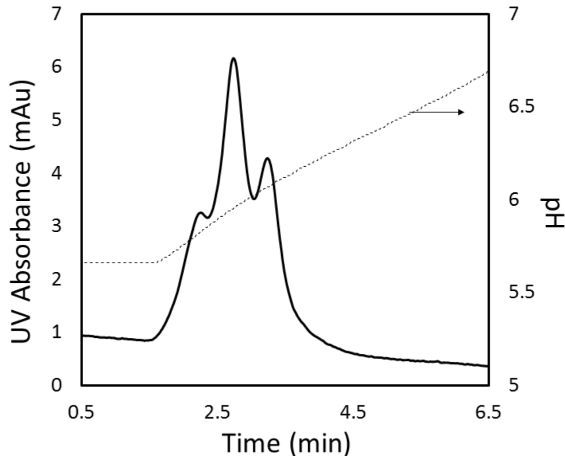

Figure 4.4: CEX analysis of hIgG1-CD4 with 1 mL LFMC device using pH gradient (feed concentration: 2.38 mg/mL; loop: 100 μL; flow rate: 10 mL/min; binding buffer: 50 mM sodium phosphate, pH 5.8; eluting buffer: 50 mM sodium phosphate, pH 8.0; 70 mL linear gradient, i.e., 0% - 100%).

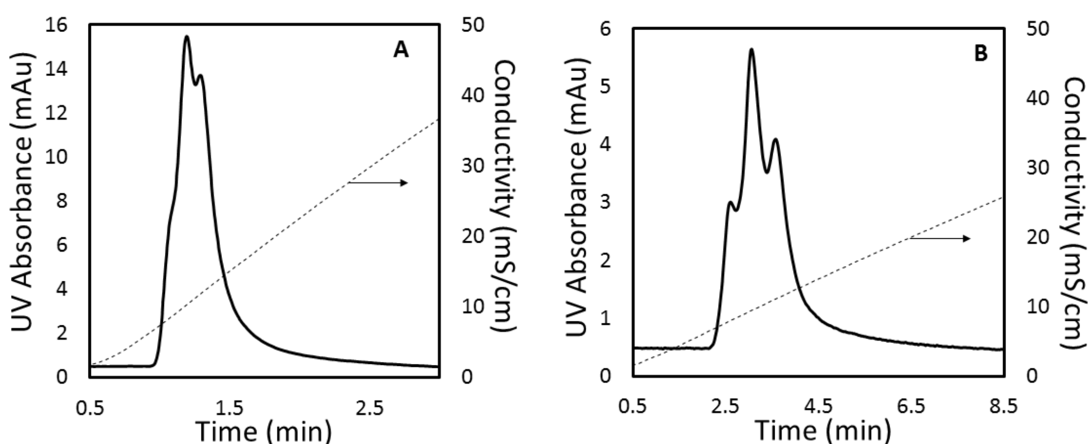

Figure 4.5: CEX analysis of hIgG1-CD4 with 1 mL LFMC device using 30 mL **(A)** and 150 mL **(B)** linear salt gradient (feed concentration: 2.38 mg/mL; loop: 100 µL; flow rate: 10 mL/min; binding buffer: 50 mM sodium phosphate, pH 5.8; eluting buffer: 50 mM sodium phosphate, pH 5.8, + 0.5 M sodium chloride).

experiments are shown in **Figures 4.3A & 4.3B** respectively. At 1 mL/min flow rate (**Figure 4.3A**) three distinct resolved peaks were obtained with in the chromatogram. This clearly indicated that the efficiency of the HiTrap™ SP HP column was significantly better than the HiTrap™ SP FF column, and variants present in the hIgG1-CD4 sample could be fractionated using this column at 1 mL/min flowrate. However, it took almost 40 minutes to carry out this separation. When the flow rate was increased to 4 mL/min (**Figure 4.3B**) variants could not be resolved.

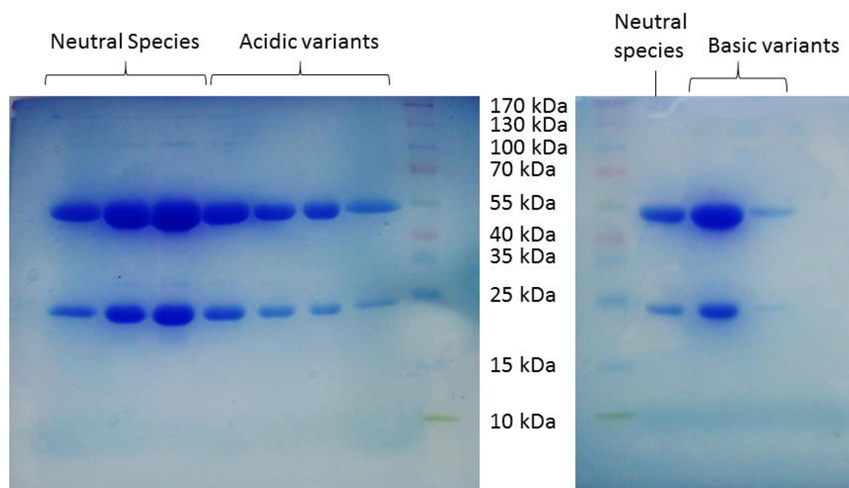

Figure 4.6: 10% SDS-PAGE (reducing) analysis of fractionated hIgG1-CD4 samples.

As the next step, we attempted to fractionate variants present in the same mAb (hIgG1-CD4) sample with a 1 mL CEX LFMC device which housed a stack of cation exchange S membranes, first using a pH gradient. In order to decrease the separation time, the flow rate was increased by a factor of 10, i.e., to 10 mL/min, and a sharper gradient (70 mL), relative to that used in the column experiments (described in the previous paragraph) was applied. **Figure 4.4** shows the chromatogram thus obtained. Three distinct peaks, similar to those obtained with the 1 mL HiTrap™ SP HP column can be seen eluting at 2.3, 2.8 and 3.3 minutes respectively. These results show that even using a flow rate 10 times higher than that used with the HiTrap™ SP HP column, it was possible to fractionate the different variant species present in the hIgG1-CD4 sample.

It has been reported that when IgG1 mAb containing sample is fractionated using a CEX column chromatography, the expected equence of elution is the acidic variants will elute

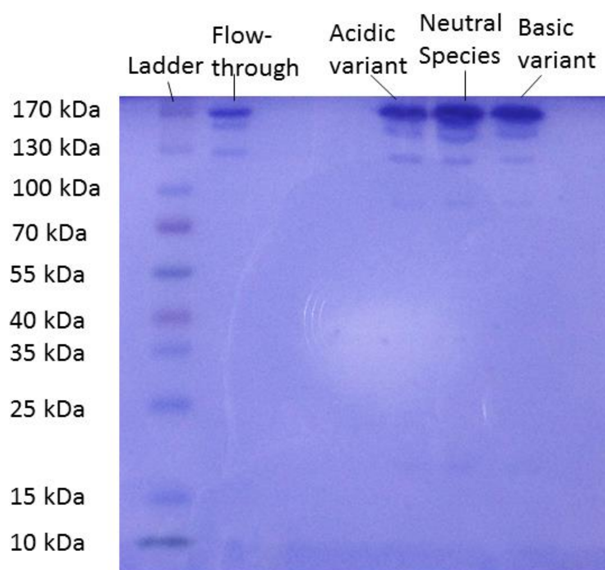

Figure 4.7: 10% SDS-PAGE (non-reducing) analysis of fractionated hIgG1-CD4 samples.

first, followed by the "neutral" main antibody fraction, followed eventually by the basic variants.[45] Based on this, it may be assumed that the three peaks at 2.3, 2.8 and 3.3 minutes respectively correspond to the acidic, neutral and basic variants present in the hIgG1-CD4 sample. Also, as reported in our previous study,[42] mAb aggregates typically elute after the mAb monomer from CEX devices. In an earlier paper[45] on the analysis of antibody variants, the presence of antibody aggregates in the fraction containing the basic variants was reported. Based on this information, it may be anticipated that hIgG1-CD4 aggregates, if any, would be present in the 3.3-minute peak.

CEX separations are more commonly carried out using salt gradients as these processes are more reproducible and a linear conductivity gradient is easier to generate than a linear pH

gradient. **Figure 4.5** shows the results obtained in hIgG1-CD4 variants separation using two different salt gradients at a constant pH value of 5.8. When a steeper salt

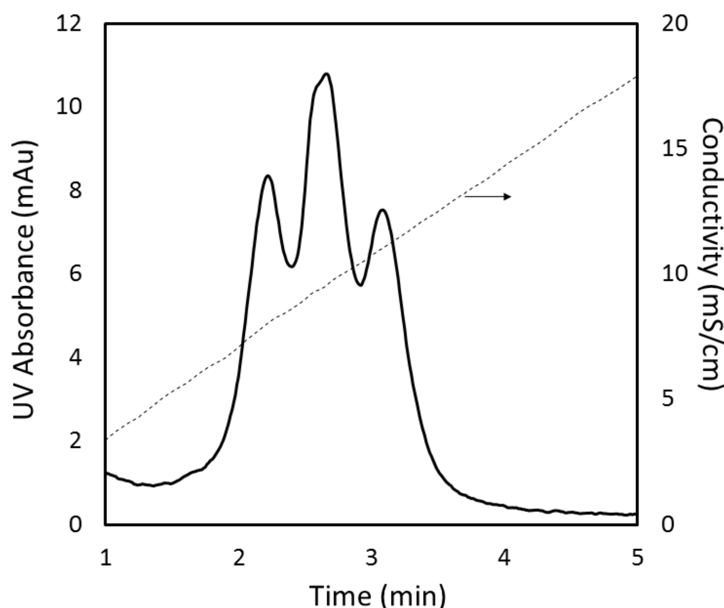

Figure 4.8: Preparative CEX separation of hIgG1-CD4 with 4.96 mL LFMC device using salt gradient (feed concentration: 0.5 mg/mL; loop: 2 mL; flow rate: 25 mL/min; binding buffer: 50 mM sodium phosphate, pH 5.8; eluting buffer: 50 mM sodium phosphate, pH 5.8, + 0.5 M sodium chloride gradient: 300 mL linear).

gradient (i.e., 30 mL) was used (**Figure 4.5A),** the variants could not be completely resolved, but the use of a shallower gradient (i.e., 150 mL) resulted in much better separation, which was comparable to that obtained using a pH gradient. It has been previously reported that IgG binds onto CEX adsorbents through the Fab region.[51] The chromatograms obtained thus far showed successful fractionation of antibody molecules

into three distinct peaks thereby confirming differences in the Fab region of the hIgG1-CD4 molecule due to various chemical degradation pathways.

Very often an antibody molecule breaks down into its constituents, i.e., heavy chain, light chain, $F(ab')_2$, Fab and Fc fragments. The binding and elution attributes of these antibody fragments on CEX media could be quite different from that of the intact antibody molecule.[51] To confirm that the peaks seen in **Figure 4.5B** were indeed variants of intact hIgG1-CD4, reducing and non-reducing SDS-PAGE analysis was performed with samples corresponding to the three peaks.

In order to obtain sufficient sample for gel electrophoresis, 2 mL of 2.38 mg/mL hIgG1-CD4 solution was loaded onto the 1 mL LFMC device, and a longer salt gradient (400 mL) was employed for elution. Samples were collected every 30 seconds during this experiment. For SDS-PAGE analysis under reducing condition (see **Figure 4.6**), representative fractions were tested while for SDS-PAGE analysis under non-reducing conditions (see **Figure 4.7**), fractions corresponding to the peaks were pooled together. The results shown in **Figure 4.6** and **Figure 4.7** confirm the absence of antibody fragments in all the samples tested. Thus, the three peaks seen in **Figure 4.5B** were clearly due to the different oxidation variants and not due to the fragmentation of the antibody molecule.

As our next step, we attempted to scale-up the separation process developed with the 1 mL CEX LFMC device using a 4.96 ml LFMC device containing the same membrane. The flow rate was increased to 25 mL/min and a 300 mL linear gradient using 0.5 M NaCl as eluent was used. A 2 mL sample loop was used to inject the hIgG1-CD4 feed material, its

concentration being 0.5 mg/mL. The results thus obtained are shown in **Figure 4.8**. Consistent with the results obtained with the 1 mL LFMC device, three distinct peaks were obtained with the 4.96 mL LFMC device.

The results discussed above clearly demonstrate that cation exchange LFMC is suitable for both analytical and preparative separation of monoclonal antibody charge variants. The resolution obtained with LFMC device was comparable to that obtained with HiTrap™ SP HP column. However, the speed of separation was an order of magnitude faster. Therefore, the cation exchange LFMC method combined both high-speed and high-resolution, a very desirable set of attributes in a bioseparation method. The high separation efficiency of the LFMC device in comparison to the resin columns could be clearly explained in terms of the greater number of theoretical plates per unit bed height (see **Table 4.1**).

4.5. Conclusions

Monoclonal antibody of the IgG1 subclass readily undergoes chemical modification and forms charged variants. The hIgG1-CD4 mAb used in our study underwent significant extent of modification during prolonged storage, resulting in a charge variant containing heterogeneous mixture. These variants could not be separated using 90-micron cation-exchange resin-based column (i.e., HiTrap™ SP FF). While a 34-micron cation exchange resin-based column (HiTrap™ SP HP) was found to be suitable for fractionation, this was only possible at a low flow rate (i.e., 1 mL/min). At this flow rate the separation was slow

and a typical run took about 40 min. Increasing the flow rate resulted in total loss of resolution in the separation of the variants.

Laterally-fed membrane chromatography or LFMC using cation exchange S membranes was examined as an alternative to resin-based chromatography. Using this technique, antibody variants could be fractionated at a 10 times higher flow rate i.e., 10 mL/min, the separation being achieved in less than 5 minutes. The LFMC-based mAb variants separation could be carried out using both pH and salt gradients. The higher separation efficiency of the LFMC device in comparison to the resin column could be explained in terms of theoretical plate height. Furthermore, the results from denaturing and non-denaturing SDS-PAGE experiments showed that the variants consisted of intact IgG1 molecules and did not contain any antibody fragments.

The separation could be scaled up using a 4.96 mL LFMC device which gave similar resolution (to that obtained with the 1 mL LFMC device) when operated at a flow rate of 25 mL/min. A point to be noted is that the performance of LFMC is also dependent on the characteristics of membrane used. Sartobind® S membranes employed in the LFMC device has a pore size of greater than 3 µm. Using strong cation exchange membranes with smaller pore size would improve peak resolution but at the same time would restrict the flow rate due to high backpressure and thereby increase the analysis time. Additionally, the separation achieved in this work can only provide information about the differences in charge on the variants. Orthogonal chemical and instrumental techniques would be required to identify what specific changes led to variants formation, e.g., oxidation, deamidation, or indeed any other kind of chemical changes.

4.6. Acknowledgements

We thank the Natural Sciences and Engineering Research Council (NSERC) of Canada, and the Ontario Research Fund – Research Excellence (ORF-RE) for funding this project. We thank the members of the Therapeutic Antibody Center, Oxford University, UK for donating the hIgG1-CD4 mAb used in this study. We thank Paul Gatt for fabricating the devices based on design provided by RG.

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CHAPTER 5: CONCLUSION AND FUTURE PERSPECTIVES

5.1. Conclusion

This technique has highlighted different analytical techniques in bioprocessing that target protein quality attributes to ensure a final uniform product. Overall, these techniques have the potential to raise awareness in the bioprocessing and biotechnology industries regarding alternative technologies that can be leveraged to generate meaningful and crucial data. However, the results presented in this thesis can provide maximum benefits only if outlooks and future perspectives are also discussed.

Firstly, the topic of protein aggregation was discussed. It is well-known in the bioprocessing community that valuable efforts and resources must be invested in treating protein feeds to obtain a pure and finished final protein product. Despite thorough processing, the loss of protein mass – primarily due to aggregation – is worrisome. Such losses translate to higher costs due to the need for additional processing, the loss of valuable protein material, and the human efforts required to recover valuable protein from aggregates. This thesis presented an alternative thermal-cycling method for recovering functional proteins from aggregated masses, such as Inclusion bodies.[1] As opposed to the tedious processing[2-4] that is currently performed, the thermal-cycling method simply involves exposing protein aggregates to rapid changes in temperature for a specified time. These temperature fluctuations disrupt the forces holding the protein aggregates together and simultaneously refolds the isolated protein chains into the appropriate three-dimensional structures.[1,5]

In the past, similar thermal-cycling of antibody aggregates has proven effective in breaking down higher-ordered protein structures into monomers and dimers.[5] This thermal-cycling technique is still in the nascent stages of development and application, and more in-depth testing is required to determine its wide-scale applicability to other aggregates. Such testing does not require copious amounts of time and effort, as the feed (i.e., protein aggregates) are almost always produced during recombinant expression and purification processes. An advantage of this thermal-cycling technique is the availability of information on the melting temperatures of proteins, which provides a good reference point for selecting thermal-cycling temperature ranges.

The proposed thermal-cycling technique can also be highly useful in *in situ* protein folding studies with fluorescent tags. A number of recombinant proteins are expressed along with fusion tags to enhance the selectivity and speed of purification.[6-8] At the same time, fluorescent tags are also incorporated into the protein chains to monitor correct protein expression.[9,10] Therefore proteins with fluorescent tags can also be subjected to thermal cycling to obtain information about the proper expression and folding of the produced protein since it would be easier to simply monitor its fluorescence.

One more application of this thermal-cycling technique worth exploring is its use in affinity studies to determine the strength of protein-protein interactions. In such studies, standard proteins such as biotin-streptavidin systems or their derivatives are used due to the high affinity produced by the noncovalent interaction between them.[11] This strong binding is the basis for many diagnostic assays that require the formation of strong and irreversible bonding between biological macromolecules. The proposed thermal-cycling technique can

be applied to test the structural rigidity or robustness of these structure of these proteins in order to obtain high- temperature structural information about them.

Secondly, this thesis examined the structural stability of proteins. All proteins undergo chromatographic processing during manufacturing, but few studies have examined the effects of the contact time between proteins and the chromatographic media. The work presented in this thesis filled this research gap by clearly showing that the strength of the protein-substrate interaction increased with time after the injection of the protein. At the same time, it was also observed that the protein underwent structural changes during the test period, which was only 10-15 minutes in duration. The result of this work brings into focus an important parameter that is often overlooked during large scale processing, namely the interaction time between the protein and substrate. However, the study presented in this thesis only accounted for analytical-scale protein testing.

In very large-scale processing, the interaction times between proteins and the separation media is often in the range of a few hours, as it takes a considerable amount of time to completely load the chromatographic column, which is then followed by a holding or interaction period. These long run times creates variability in the product quality because the protein molecules that are introduced first into the chromatographic column remain in contact with the separation media for a longer time compared to those that are introduced at the very end of the loading step.

Several conclusions can be drawn from the above study. Perhaps the most significant conclusion is the necessity of reworking, the entire process of entire protein-loading and

elution to ensure uniform interaction between the proteins and the separation media. Next, the findings of this study also indicate that the feed solution introduced into the chromatographic columns should contain appropriate additives or stabilizers to prevent the proteins from denaturing or unfolding during processing. Additionally, stricter quality checks should also be put in place to monitor the quality of proteins during manufacturing.

Lastly, this thesis highlighted the importance of heterogeneity in proteins, and especially with antibodies. There are several instances where the native form of protein consists of 2 separate but functional species, for example conalbumin and hemoglobin. However, for some proteins, like therapeutic monoclonal antibodies, it has been noted that during manufacture, processing, and storage, many changes take place on the surface in the form of hydrophobicity,[12,13] surface charge,[12] isoelectric point (pI),[14-19] and local confirmation.[20,21] This results in the final pool containing several different variants of the same protein molecule. Thus, heterogeneity in antibodies also affects the final structure of individual protein chains. Prior studies have found that, when a dose containing antibody variants is injected into test specimens, the variants showed different binding capabilities with a noticeable effect on antibody half-life in serum.[22,23] Additionally, the presence of inappropriately glycosylated protein has resulted in a lower half-life and, in some cases, has also induced allergic reactions.

Overall, the presence of charged variants of proteins is certainly not beneficial for commercial and therapeutic use. Although there are several techniques that can be used to successfully identify and separate protein charge variants, these techniques are tedious and cumbersome to operate. Therefore, the use of LFMC to identify and fractionate variants is

a positive development, as it expands the possible applications for this new technology. Not only is the LFMC technique able to identify the charged variants in a given mixture, but it is able to provide results more quickly (i.e., less than five minutes) while using smaller bed volumes of (approximately 1 mL). The LFMC technique can be used to explore multiple avenues where high-resolution and faster results are required for quick quality tests, as bioprocessing usually entails large-scale operations which have the potential to generate variants of the protein being manufactured. Therefore, operators can implement the LFMC technique to quickly assess the quality of protein being produced and to guide the implementation of appropriate corrective measures, if necessary.

In addition to the monoclonal antibody charge variants, other process- and product-related impurities also end up in the cell supernatant thereby adding complexity to the already challenging problem of monoclonal antibody purification. Handling the supernatant feed will be relatively ease if impurities such as endotoxins and viruses are removed in the early processing steps thus easing the burden on downstream purification. However, virus filtration is performed towards the end of protein purification steps where the protein is highly pure and fairly concentrated. This presents a problem which needs careful consideration and planning since viruses are most difficult contaminants to detect and remove when working with mammalian cell lines and often require microscopy techniques for their detection. The main source for the origin of these viruses are the cell lines themselves that are known to contain endogenous retroviruses.[24-26] Additionally, viruses may also get introduced into the culture when using different media components.

Currently observed best practice approach relies on selection of the cell lines that are free from viral contaminants, selection of raw materials with low risk of introducing viruses, and incorporation of steps to remove viruses during protein purification.[27] Nonetheless, the risk of virus infection in the cell culture fluid is real that can cause adverse effects. Therefore, virus removal by filtration is a common practice in the industry and has become a standard approach during manufacture of therapeutic proteins. This stringent condition has been put forth by the regulatory agencies such as the US Food and Drug Administration (FDA) which requires removal of viruses and viral particles from protein drug formulations.[28]

Quite commonly, filtration membranes are used to remove viruses and virus particles from feed containing monoclonal antibodies. Virus removal from contaminated feed using filters works on size-exclusion mechanism whereby the large-sized viruses are retained on the membrane surface and relatively small antibody molecules pass through the filter pores. However, during downstream processing the macromolecular proteins tend to self-associate, form aggregates, or potentially interact with the polymeric filtration surfaces thereby causing membrane fouling. This fouling can significantly impact the efficiency of virus removal.[29-31] Especially, when the feed contains charge variants of monoclonal antibodies during virus filtration, it can further complicate the filtration process since variants could interact differentially with the ligands on membrane. In general, protein variants with higher net surface charges could potentially strongly adsorb in the membrane pores and initiate fouling mechanisms either by pore constriction, and partial or complete pore blockage.

The problem of membrane fouling because of the presence of protein charge variants therefore demands careful analysis of the processing steps.[32] This can be tackled by using membrane chemistries wherein the pendent ligands inside membrane pores during virus filtration do not present charged sites where protein variants can bind. Alternatively, a membrane adsorber could be used to remove product- and process-related impurities such as DNA, host cell proteins (HCPs) and protein aggregates that can be eliminated in the flow-through mode thereby retaining only the target protein.[33] In this way significantly pure feed containing only the antibody and trace virus particles can be achieved which then can be processed using a virus filter to remove viruses present in the solution alongside monoclonal antibodies.

Since there is a significant shift in focus from batch processing to continuous processing in manufacture of monoclonal antibodies, various changes are being explored to identify critical processes and eliminate the undesired steps to reduce overall cost.[34,35] Such changes ensure smaller and more efficient unit operations. For the removal of monoclonal antibody charge variants and virus filtration, it will be a great challenge to streamline both the operations and ensure seamless processing. This is because traditional virus filtration is performed as a batch unit operation wherein the feed contains uniformly mixed solution. On the contrary, when operating in a continuous mode, this same feed coming into virus filtration stage can vary significantly over time in terms of its composition.

Likewise, when considering the scenario of a continuous operation in the manufacture of monoclonal antibodies, the challenge will be to identify and fractionate protein charge variants from the normal antibody variant. Currently used large sized packed columns for

ion exchange chromatography of monoclonal antibody feed are operated under the bind and elute conditions. These columns offer the disadvantage of a slow diffusive mass transfer process. In packed columns the uniformity in fluid distribution and even distribution of protein feed can be never achieved. This problem gets amplified when the process is further scaled up. Working with hundreds and thousands of liters of protein feed using ion exchange columns will eventually lead to a heterogeneous stream of monoclonal antibody molecules in the eluate. This is because the entire capacity of the packed column will be used up in considerable amount of time and the interaction between protein and column media will not be uniform in terms of the contact time.

Therefore, such operations which involve binding and elution of proteins will be difficult to process under continuous mode. However, the impact on monoclonal antibody can be minimized if the process can be accelerated by use of membranes wherein protein mass transfer primarily occurs due to convection. The data presented in this thesis using LFMC technique has clearly demonstrated that by novel design of membrane devices, the processing time of an ion exchange step can be shortened to few minutes at an analytical scale. The data presented is just a proof of how challenges faced in column chromatography can be overcome by use of membrane chromatography. There is still a long way to go before membrane chromatography can be fully integrated into downstream purification processes due to inherent challenges facing membrane chromatography.

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